Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 80 MAY, 1952 No. 1

SECTION MEETINGS

CLEVELAND, O.	
University Hospitals	March 12, 1952
IOWA	
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University of California, Los Angeles	April 3, 1952
SOUTHEASTERN	
Emory University	January 17, 1952

Mechanism of Hypoglycemia Responsiveness in Relation to Changes in Blood Eosinophile Level.* (19500)

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In the normal individual the intravenous administration of a small dose of insulin causes an abrupt decline of the blood sugar concentration which is followed within 20 to 30 minutes by a sharp rise. This phenomenon is considered to be a result of stimulation of a homeostatic mechanism for restoration of normal blood sugar levels and not of the cessation of the activity of the injected insulin. In previous investigations we demonstrated that the moderate hypoglycemia caused by the intravenous administration of 0.1 unit of crystalline insulin per kg of body weight in normal individuals is accompanied by a significant rise of the absolute lymphocyte count with the peak at 45 to 90 minutes after the insulin administration(1). In a subsequent report it was shown that the mean percentage increase of the absolute lymphocyte count above the fasting value in diabetic patients given the same insulin dosage declined with increasing degrees of impairment of hypoglycemia responsiveness(2). Moreover, 2 diabetic patients in whom there was no evidence of counter-regulatory response to the falling blood sugar level in the Insulin Tolerance Test, failed to show a rise in the absolute lymphocyte count. This suggested that the same mechanism which is responsible for the rise of the absolute lymphocyte count is also responsible for antagonizing the action of insulin on the blood sugar level.

The present study was undertaken with the objective of elucidating the mechanism of the lymphocytosis after insulin and at the same time it was hoped to clarify the trigger mechanism for hypoglycemia responsiveness. Since

* Supported by a grant from the National Institutes of Public Health, U. S. P. H. S.

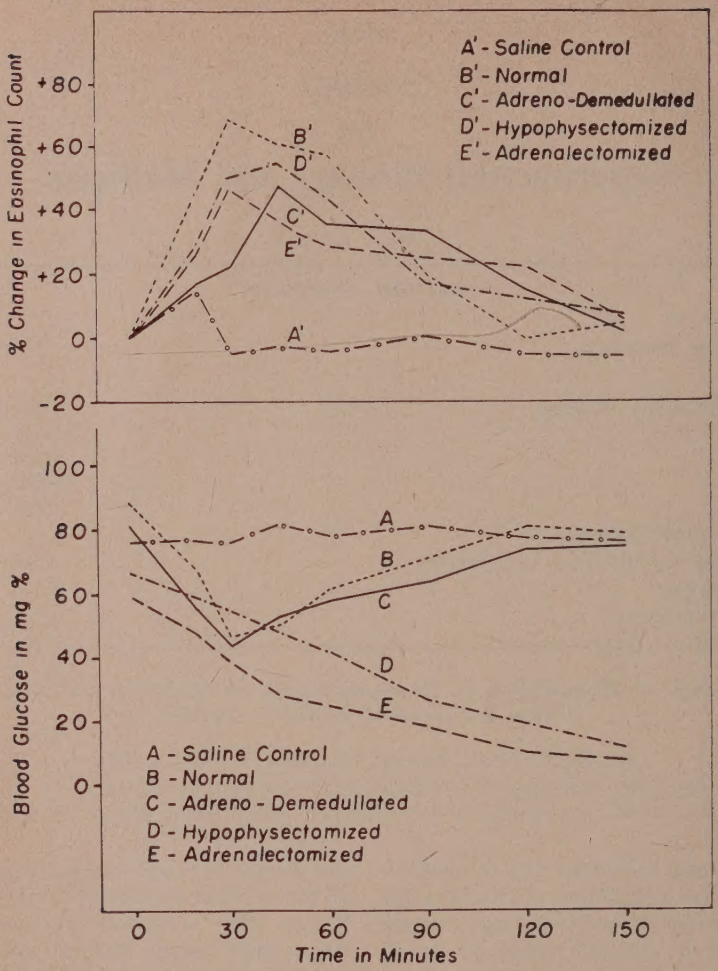

FIG. 1. Mean blood sugar levels and mean percentage change of the eosinophil count after intravenous injection of insulin in normal, adreno-demedullated, adrenalectomized, and hypophysectomized dogs compared with saline controls.

it was observed that the change in the eosinophil level paralleled the change in absolute lymphocyte count, for technical reasons this response was studied rather than the lymphocyte response(3-6).

Material and methods. The study was carried out on normal, adreno-demedullated, bilaterally adrenalectomized and hypophysectomized mongrel dogs weighing from 15 to 20 kg. A 125 mg Percorten tablet (Ciba) was inserted subcutaneously immediately postoperatively in all adrenalectomized animals. Both the adrenalectomized and hypophysectomized animals were maintained on normal saline in addition to the regular diet of canned dog food and table scraps. The experimental animals were divided into 4 groups: 1. 10 normal controls; 2. 5-adreno-demedullated dogs; 3. 3 hypophysectomized dogs; 4. 5 adrenalectomized dogs.

The experiments in the operated animals were carried out after the 14th post-operative day and were repeated on the same dog at 2-week intervals. After a 24-hour fast and

2 hours after the induction of intraperitoneal nembutal anesthesia, 0.1 unit of crystalline insulin (Lilly) per kg of body weight was injected intravenously. When necessary, as indicated by a returning corneal reflex, the animals were given additional small doses of anesthesia. Furthermore, 6 control experiments using saline instead of insulin were carried out. Duplicate venous blood samples were collected before and at 20, 30, 45, 60, 90, 120, and 150 minutes after the injection of insulin. The blood sugar determinations were done on serum by the Nelson modification of the Folin-Wu micro method(7) and the eosinophil counts were done according to the procedure described by Randolph(8). The change of the eosinophil level was expressed in terms of the percentage change from the fasting value which was taken as 0%. The experiments were started 2 hours after the induction of anesthesia since it was found that in the untreated normal dog this time interval allowed for recovery from the initial stress incident to anesthetizing the animal.

Results. Both the blood sugar and the eosinophil level of the saline control animals showed a non-significant variation from the fasting level (Fig. 1, Curve A, A^1). In the intact animal the lowest blood sugar after insulin was reached within 20, 30, or 45 minutes whence it rose gradually. This was accompanied in all instances by a significant rise in the eosinophil level which reached its peak at 30 to 60 minutes and was then followed by a gradual decline. The mean values are shown in Fig. 1 (Curve B, B^1). The response of the blood sugar level and of the eosinophil level of the adreno-demedullated animal after insulin administration was in all respects similar to that of the intact dog (Fig. 1, Curve C, C^1). In the hypophysectomized and the adrenalectomized animals the fasting blood sugar level was depressed and after injection of insulin it declined progressively with no evidence of hypoglycemia responsiveness (Fig. 1, Curve D, E). However, the eosinophil level in both groups, on the other hand, showed a significant rise which was similar to that of the normal (Fig. 1, Curve D^1, E^1).

Discussion. Cannon *et al.*(9) showed that hypoglycemia causes a release of epinephrine. In view of the glycogenolytic action of the medullary secretion it has been assumed that hypoglycemia responsiveness is based on a stimulation of the adrenal medulla(10,11). However, Zucker and Berg(12) demonstrated that the adreno-demedullated animal has a normal response to insulin. This has been confirmed by our experiments. Furthermore, the rise of the eosinophil level in the adreno-demedullated animal is similar in all respects to that of the normal, indicating that this response is not mediated by activation of the adrenal medulla.

However, it is generally agreed that in the absence of the hypophysis or the adrenal there is a failure of hypoglycemia responsiveness (13,14). This has led to the hypothesis that an immediate response of the pituitary-adrenal axis is the mechanism for hypoglycemia responsiveness. This viewpoint is supported by the finding that there is a decline of adrenal cholesterol and ascorbic acid as a response to a lowered blood sugar level, indicating a release of glucocorticoids(15).

Since in both the hypophysectomized and adrenalectomized animals the rise of the absolute eosinophil count after insulin was similar to that in the normal, it can be assumed that this response is not effected by a change in activity of the anterior pituitary or the adrenal cortex. As previously stated, the mechanism for the rise of the eosinophil level is probably identical with that of hypoglycemia responsiveness. Therefore, it can also be inferred that an immediate response of the adrenal cortex and the anterior lobe of the pituitary are not a necessary component of the homeostatic mechanism even though the hypophysectomized and adrenalectomized animals are hypoglycemia unresponsive. This is supported by a previous hypothesis(16) that the failure of hypoglycemia responsiveness which is observed after removal of the pituitary or adrenal glands may be primarily the result of depletion of the glycogen stores of the liver rather than the destruction of the mediating mechanism.

Summary. 1. The intravenous administration of 0.1 unit of crystalline insulin/kilo body weight to the intact anesthetized dog caused

an abrupt decline of the blood sugar level which is followed within 20 to 45 minutes by a sharp rise. This is accompanied by a significant rise of the eosinophil level which is thought to be mediated by the same mechanisms as is responsible for the rise of the blood sugar level. 2. The adreno-demedullated animal shows normal hypoglycemia responsiveness in the insulin tolerance test and an accompanying rise of the eosinophil level similar to that of the intact animal. This indicates that the medullary secretion is unnecessary for either of these phenomena. 3. In the adrenalectomized and hypophysectomized animals a rise of the eosinophil level similar to that of the normal was demonstrated after insulin, even though the blood sugar changes showed no evidence of hypoglycemia responsiveness. This is interpreted to signify that the mechanism for hypoglycemia responsiveness in the hypophysectomized and adrenalectomized animal is intact but is not apparent, due to depletion of liver glycogen in the absence of the anterior-pituitary and adreno-cortical secretions.

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Received May 1, 1952. P.S.E.B.M., 1952, v80.

Autonomous Transplantable Pituitary Tumors Arising in Growths Dependent on Absence of the Thyroid Gland.* (19501)

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"Invasive," "autonomous," "unrestrained" are adjectives commonly used to characterize cancerous growth. The study now to be described indicates the uncertainty of these terms in relation to neoplastic growth. Autonomy may be only apparent or partial, can never be certified to be absolute, and metastases may occur with conditioned neoplasms. Destruction of the thyroid by I^{131} (250-300 μc) is followed by the development of pituitary growths 10 to 14 months later(1). These growths can be grafted readily in the muscle of mice similarly radio-thyroidectomized but not in normal mice(2). They grow progressively in the former until the death of the host and almost invariably metastasize to the draining lymph nodes. In over 100 such tumor-bearing mice not a single spontaneous regression was noted.

The procedures of inducing and transplanting the pituitary tumors have been described (2). All studies have been made in C-57 black mice. Seven transplantable strains were established thus far in radio-thyroidectomized

* Work performed under Contract for the Atomic Energy Commission.

TABLE I. Transplantation of Original I^{131} Induced Pituitary Growths.

Original Mouse No.	I^{131}, μc subcut.	Days from inj. to death	Recipients: I^{131} pretreated: No. + / No. inj.	Recipients: I^{131} pretreated: Latency, days	Recipients: Normal: No. + / No. inj.
B 3	265	536	7/7	(61-158) 117	—
19	198	516	2/2	(157-175) 166	0/3*
77	275	476	3/10	(156-232) 188	—
163	±400	501	7/9	(183-251) 220	0/10
124	±400	474	3/4	(117-283) 161	0/5†
101	±400	474	12/12	(111-275) 153	0/2
82	278	564	2/5	(176-359) 268	0/7
		Total	36/49		0/27

* Gonadectomized. † Two of these were gonadectomized.

TABLE II. Origin and Passages of an Autonomous Pituitary Tumor Line (B3).

Passage	Sex	Mice: I^{131} pretreated	Mice: Normal	Latency period (days): I^{131} pretreated: Range	Latency period (days): I^{131} pretreated: Avg	Latency period (days): Normal: Range	Latency period (days): Normal: Avg
I a	♀	13/14*	8/8*	80–173	96	179–239	208
IIa	♂	3/4	8/8	59–76	68	38–60	45
b	♂	3/4	9/10	45–53	50	45–63	49
	♀	—	10/10	—	—	29–63	41
c	♂	0/15	6/9	—	—	47–67	54
	♀	—	3/4	—	—	67	67
d	♂	0/4	5/5	—	—	46–56	51

* Number of mice with "takes" over number inj.

hosts (Table I). From 474 to 564 days elapsed after injection of I^{131} until a marked loss of weight, humped posture, ruffled fur, and cachexia suggested the presence of a pituitary growth. The animals were killed and fragments of the pituitary growths were grafted into the muscle of 49 radio-thyroidectomized mice, in 36 of these with success. Grafts attempted simultaneously in 27 normal mice were uniformly unsuccessful. The period of latency of the growth in the primary grafts was usually long, averaging 117 to 268 days in these 7 strains (Table I). The first generation grafts grew very slowly and measured but 1 to 2 cm across after 2 to 4 months of manifest growth when the animals were sacrificed to make further subpassages. Several of these I^{131} pretreated recipients had their own primary pituitary tumors at death. In the 2 strains in which primary grafts were made only in radio-thyroidectomized mice, subpassages were also made in normal mice thus far without success.

In one subpassage of the first strain (B3) so established in I^{131} pretreated mice, the grafts grew also in normal mice (Tables II and III), but appeared after a longer latency period; this averaged 96 days in radio-thy-

TABLE III. Transplantation of Pituitary Growths of Strain B3 Dependent Line and Origin of an Autonomous Line in Passage Ia.

Passage	I^{131} pretreated hosts: No. + / No. inj.	I^{131} pretreated hosts: Latency period, days*: Range	I^{131} pretreated hosts: Latency period, days*: Avg	Normal hosts: No. + / No. inj.	Normal hosts: Observ. period, days
Original	7/7	61-158	117		
I a	13/14	81-172	95	8/8†	319
b	10/10	95-104	96	0/11	240
c	11/11	96-190	116	0/11	246
d	6/6	77-96	87	0/6	210
II a	7/7	93-139	125	0/7	225
b	6/6	93-139	118	0/7	225
c	6/6	65-75	73	0/5	197
d	6/6	74-100	85	0/4	188
e	15/15	61-126	89	—	—
f	4/4	90-112	98	0/4	159
g	5/5	72-99	82	0/11	126
h	5/5	77-91	83	0/5‡	113
IIIa	10/10	60-76	68	0/16	88

* In I^{131} pretreated hosts.
† Origin of the autonomous line.
‡ Received 25 μc of I^{131} that does not destroy the thyroid gland.

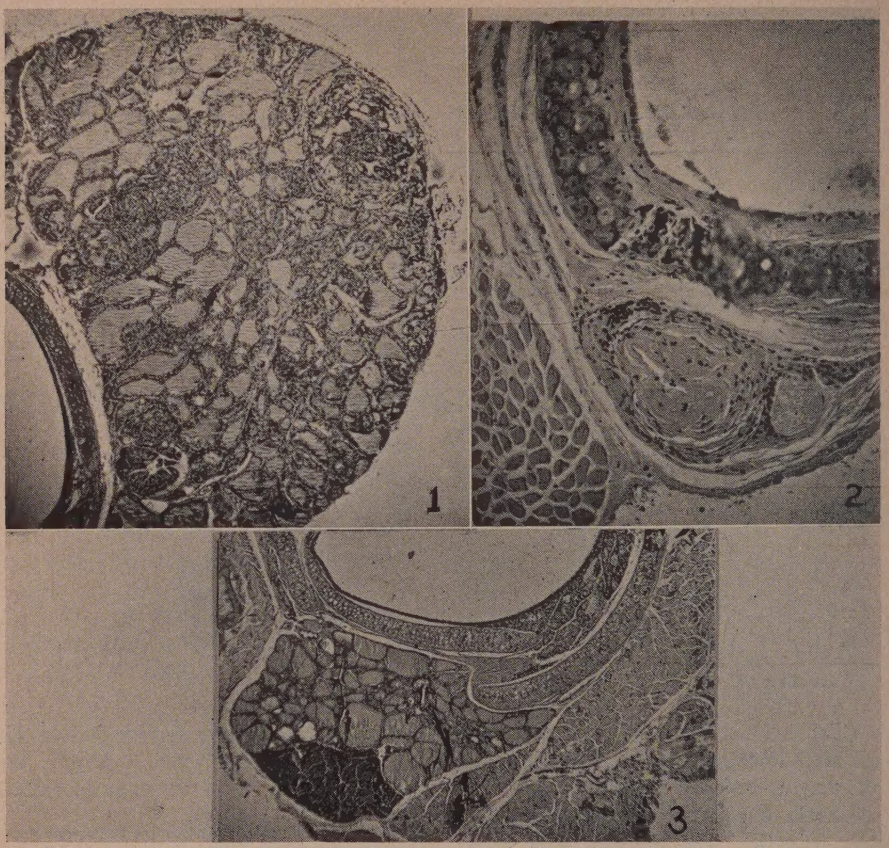

FIG. 1. Thyroid with numerous nodular areas of hyperplasia (adenomas). Mouse carried a pituitary tumor of the autonomous line. × 40.

FIG. 2. The site of a thyroid destroyed by I^{131}, marked by "radiation arteritis." Mouse carried a grafted pituitary tumor of the dependent line. × 100.

FIG. 3. Normal thyroid gland of a mouse with parathyroid gland. × 40.

roidectomized mice and 208 days in normal mice. Similarly, the tumors grew at a much slower rate in normal mice than in those receiving thyroid destructive doses of radioiodine. In further subpassages of this autonomous line the tumor grafts took even more readily in normal mice than in radio-thyroidectomized mice as indicated in Table II. Passage IId includes 4 additional mice (not listed in Table II) pretreated with thiouracil beginning 13 days before grafting. In 2 of these mice tumors appeared after 45 days.

Table II indicates the readiness with which the pituitary tumor that gained autonomy can be passed to normal mice. In contrast, other passages of strain B3 in which this autonomous line originated remained dependent on the absence of the thyroid gland as indicated in Table III during the period of observation.

Discussion. It is remarkable that transformation should occur in the first subpassage and not again during the next 11 subpassages involving 87 normal mice receiving tumor fragments.‡ In contrast all 91 radio-thyroidec-

‡ After the observation period indicated, several months after death of the I^{131} pretreated mice with large tumors, some of the normal mice have developed small slowly growing tumors. This phenomenon is being studied.

tomized mice that had been injected with the same material developed tumors and almost all metastasized to the regional lymph nodes. The passages were made with tumor fragments. It is possible that some pituitary cells in lymph nodes did acquire some autonomy.

The salient characteristic features of this autonomous in contrast to the dependent growth† are as follows: Hosts bearing autonomous growths have tremendously stimulated thyroids (Fig. 1). In I^{131} pretreated hosts of the dependent line the thyroids are absent (Fig. 2) or atrophic, non-responsive to TSH. The thyroid in the former mice is several times larger than in normals (Fig. 3); the colloid tends to disappear and numerous adenoma-like nodules replace the normal gland (Fig. 1).

There is morphologic evidence of stimulation of the gonads in mice of both sexes with both FSH and LSH in both autonomous and dependent lines. This stimulation is, however, less intense in the autonomous line.

Hyperplasia with cystic dilatation of the extrahepatic ducts has not been seen in mice with autonomous pituitary tumors thus far autopsied. This endocrine effect occurred in most radio-thyroidectomized mice bearing large tumors(3). Most autonomous tumors thus far studied were, however, smaller than the dependent tumors, but many of them equaled in size the dependent tumors which were associated with common duct cysts. Further observations are needed to ascertain if the absence of stimulation of the common duct is related to the tumor size, lack of the thyroid gland of the hosts or differences in the tumor cells.

The adrenals in both lines are smaller than normal or approximately normal. There are retrogressive changes in the reticular zone of the adrenal cortex in both tumor lines. In the autonomous line the cells of the reticular zone characteristically contain large (presumably fat) vacuoles. Although in radio-thyroidectomized mice the thymus glands were characteristically atrophic, in normal mice with autonomous growths they were frequently present and approximately normal in size. These morphological observations suggest lack of ACTH production by these tumors. The pituitaries of these mice have thus far not been adequately studied. In the radio-thyroidectomized tumor-bearing mice, they are enlarged and show either the well-known changes of thyroidectomy or hyperplasia with adenoma-like nodules as described by Gorbman(1). The pituitaries of normal tumor-bearing animals appear of normal size and contain the normal type of cells.

Summary. 1. All of 7 pituitary growths induced by thyroid destructive doses of I^{131} proved transplantable to mice similarly pretreated but not to normal mice. In a subpassage of one strain (B3) a tumor acquired the ability to grow in normal mice giving origin to a pituitary tumor line which can be grafted as readily if not better in normal mice than in radio-thyroidectomized mice. 2. In normal hosts of this autonomous tumor there is a tremendous hyperplasia of the thyroid gland with multiple adenoma-like nodules. There is also some gonadal stimulation of the hosts but no evidence of ACTH secretion by these tumors. Thus a gain in autonomy occurred with retention of the ability of these pituitary tumor cells to secrete thyroid and gonad stimulating hormones. 3. These studies indicate the uncertainty and relativity of the term "autonomy" in relation to neoplastic growth. Regional metastases do not indicate "autonomy" as they occur also with conditioned neoplasms.

The technical assistance of Mr. W. D. Gude and Mrs. Peggy Ledford is acknowledged.

† These terms are retained for didactic purposes only.

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Received April 8, 1952. P.S.E.B.M., 1952, v80.

Transitory Variations in Serum Prothrombin Activity. (19502)

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When blood clots in the test tube, only a portion of the prothrombin is consumed. The amount of prothrombin activity remaining in the serum has recently been shown to have considerable clinical significance(1). Deficiencies in the anti-hemophilic globulin (thromboplastinogen) or the platelet-tissue thromboplastic enzyme result in less consumption of prothrombin and consequently more residual serum prothrombin activity. Quick (1) has demonstrated that serum prothrombin activity can be estimated by using deprothrombinized plasma as a source of fibrinogen. His data indicate that incubation of the whole clot may alter serum prothrombin activity quite differently from similar incubation of the separated serum. This fact is probably responsible for much of the difficulties and multiplicity of technics suggested for the estimation of prothrombin consumption(3). Quick suggests that prothrombin consumption be determined by incubating 3 aliquots of blood for various time intervals before separation of the serum and doing 7 estimations of prothrombin activity of these 3 sera after various periods of further incubation. This paper presents data describing serially the pattern of serum prothrombin activity following varying periods of incubation before and after separation of the serum from the clot. These data are sought in order to determine the conditions which will best reflect the prothrombin-consuming property of a specimen of blood. Knowledge of these conditions may make it possible to estimate prothrombin consumption reliably and reproducibly by a single determination on one aliquot of blood. Data are also presented on the use of fibrinogen in place of "prothrombin-free" rabbit plasma for determining serum prothrombin activity. A standardized, dry preparation containing fibrinogen, thromboplastin, $CaCl_2$, and NaCl in optimal amounts is employed.* The preparation is free from prothrombin, oxalate, and citrate(2) and is stable at 5°C for at least several months. This single reagent mixture is prepared for use by the addition of water, and permits the determination of serum prothrombin activity as simply as the usual plasma prothrombin time. The normal range of plasma prothrombin time with the thromboplastin employed is from 14 to 17 seconds.

A. *Effect of incubation before and after separation of serum from the clot. Method.* Three ml aliquots of blood were placed in a 37.5° water bath promptly after venipuncture. At various time intervals each aliquot was centrifuged at 3000 RPM for 5 minutes, the serum separated, and returned to the water bath. The serum prothrombin time of each aliquot of serum was estimated by adding 0.1 ml serum to 0.2 ml reagent mixture* at 37°. Estimations were made one minute after separation, and at intervals thereafter. *Data.* Fig. 1 illustrates the results obtained with normal blood. The beginning of each bar represents the time each aliquot of blood was incubated before centrifugation; the bar represents the period of centrifugation; the dot the serum prothrombin time one minute after separation of the serum; the curves the prothrombin time of each serum aliquot on subsequent incubation. There appear to be 3 phases of activity. 1) Serum separated as

* The reagent employed is a combination of thromboplastin and fibrinogen prepared as follows: Thromboplastin is first prepared as an aqueous extract of dried rabbit brain and lung tissue, and includes calcium chloride. Volumes are adjusted to give a preparation containing 0.0125 M calcium chloride and yielding a normal plasma prothrombin time of 15 seconds. Fibrinogen is precipitated from deprothrombinized oxalated beef plasma, and lyophilized to a dry state. This fibrinogen is placed in vials each containing 7 mg of clottable protein. Extract of rabbit brain and lung tissue is then added to each vial and the combination frozen and dried from the frozen state.

The authors wish to thank Dr. R. Kroc and E. White of Chilcott Laboratories, Morris Plains, N. J., for preparing this reagent.

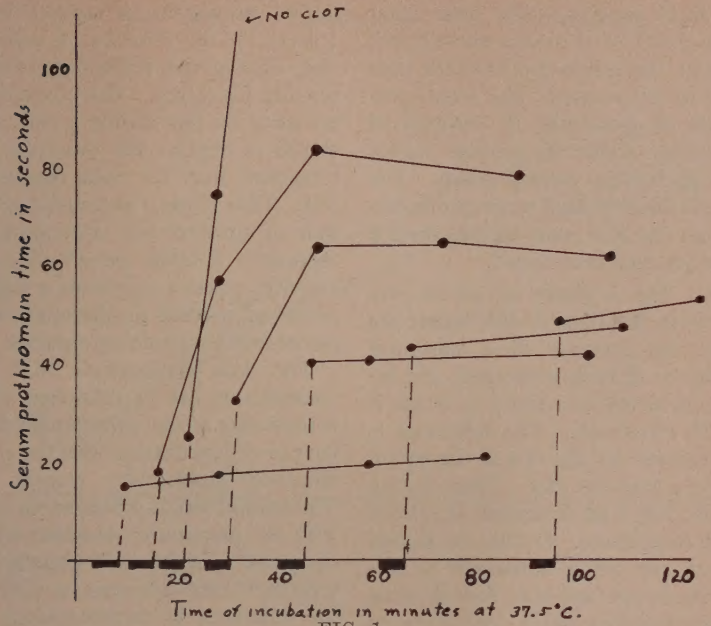

FIG. 1.

soon as normal blood clots yields an apparent serum prothrombin time equal to or shorter than that of the original plasma, and loses that activity slowly on subsequent incubation. 2) If the separation is delayed for several minutes, there is considerable loss of prothrombin activity on subsequent incubation of the serum. 3) If a period of about one hour or more is allowed to elapse before the serum is separated, the prothrombin activity of the subsequently incubated serum remains relatively constant. Reproducible, consistent serum prothrombin estimations are obtained only after this relatively stable state is reached. In spite of incubation, blood from 5 known hemophilic subjects did not go beyond the first phase of high prothrombin activity. Because of these observations, the method described below has been adopted for routine estimations of serum prothrombin time (prothrombin consumption).

B. *Comparison of serum prothrombin time of normal and hemophilic blood. Method.* Two to 3 ml of venous blood is obtained with a minimum of trauma and is introduced gently into a 12 mm diameter glass tube. The specimen is placed in a water bath at 37.5°C for one hour. It is then centrifuged at 3000 RPM for 5 minutes. The serum is separated and returned to the water bath for several minutes. The serum prothrombin time is then estimated by adding 0.1 ml of serum to 0.2 ml of the thromboplastin-calcium-fibrinogen reagent,* and determining the time necessary for clot formation with the aid of a wire loop as described for routine plasma prothrombin time determinations. The end-point is sharp and is preceded by a fine flocculation.

Data. Twenty-five normal male subjects between the ages of 18 and 25 years were used for control studies. Blood was obtained by free flow into a test tube through a 19 gauge needle. The serum prothrombin time of 24 of these subjects averaged 41.1 seconds (range from 33 to 54 seconds) with a standard deviation of 2.1 seconds. One subject, not included in the average, had a serum prothrombin time of 89 seconds. Repeated estimations of the serum prothrombin times of 5 hemophilic subjects were never above 25 seconds, and usually between 14 and 20 seconds. The serum prothrombin time of one hemophilic

subject rose to 29 seconds several hours after a transfusion of 500 ml of normal whole blood. Two days later the serum prothrombin time had returned to 19 seconds. The serum prothrombin time of chronically ill hospitalized subjects averaged about 10 seconds higher than the young healthy control group. The significance of relatively high serum prothrombin time values found in some chronic disease states has not yet been determined.

Discussion. The 3 phases of serum prothrombin activity described in this report are suggestive of the presence of a substance which develops as clotting progresses, and interferes with prothrombin activity of serum if separated with the serum. This substance is apparently removed by the clot if the serum is not separated from the clot. Thus, during the first phase, only a small amount is formed and promptly neutralized. During the second phase some of the active substance is separated with the serum, and may destroy some of the protein moieties which contribute to prothrombin activity. If the serum remains with the clot, however, (third phase), all of this substance may be removed by the clot. This sequence is consistent with the concept that the substance is thrombin, since early in coagulation, thrombin is present only in traces (phase 1). Later it develops in appreciable amounts which may be separated with the serum (phase 2). This thrombin in serum, free from its normal adsorbents (fibrinogen, fibrin, and platelets), may exert a binding or proteolytic effect on other serum proteins, and may destroy some component of the prothrombin complex. However, if the serum is permitted to remain in contact with the clot (phase 3), the thrombin is adsorbed by the clot, leaving the residual prothrombin unaltered in the serum. This concept removes the necessity for postulating a new inhibitor substance to explain the observed facts. It is consistent with the data reported by others (3). Thus phase 1 represents only the initiation of prothrombin consumption; phase 2 represents a labile period of free thrombin activity; phase 3 represents a relatively stable period of residual prothrombin, reflecting the prothrombin consuming capacity of the blood.

The data demonstrate that prothrombin consumption can be estimated adequately by determining serum prothrombin time after one hour of clot incubation with the aid of a single, relatively stable, dry reagent preparation. The normal serum prothrombin time obtained with the preparation is about 40 seconds, as compared with about 25 seconds by the Quick technic. This difference is probably due to the difference in thromboplastin preparation plus the fact that only fibrinogen, free from extrinsic accessory clotting factors, is added. Much of the clinical significance of altered prothrombin consumption remains to be determined.

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Received January 14, 1952. P.S.E.B.M., 1952, v80.

Comparison of Parenteral and Oral Protein Feeding on Radiation Susceptibility in Protein-Depleted Rat. (19503)

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The damage that is apparent histologically in the intestinal epithelium following radiation has stimulated several investigations of intestinal absorption following radiation. Meed, Decker, and Bennett found no marked impairment of fat absorption from the intestine due

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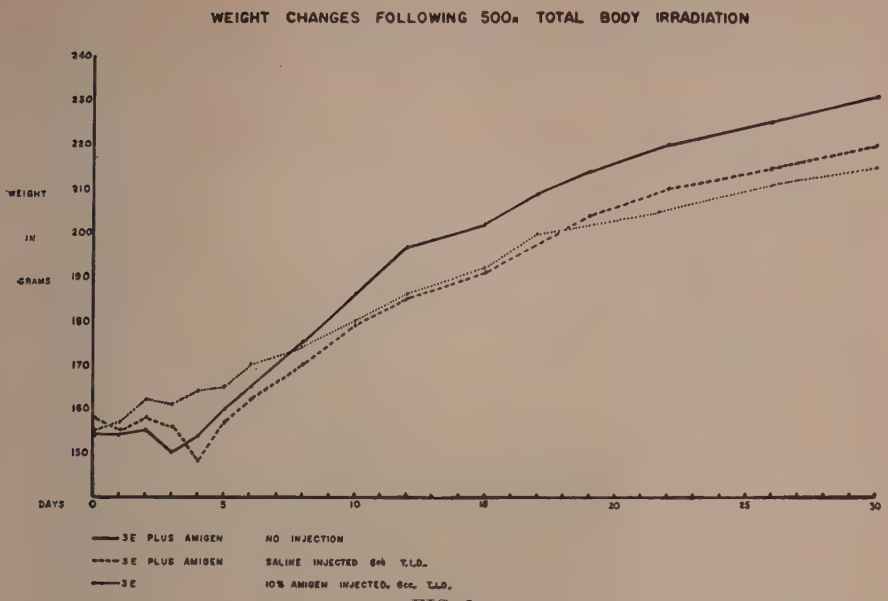

FIG. 1.

to radiation(1) though earlier investigators reported decreased fat absorption in dogs following radiation(2). Buchwald reported that the rates of absorption of glucose, fructose, and mannose were markedly diminished in rats 20 and 40 hours after sublethal radiation (3). These observations were recently confirmed by Barron(4). Since this present experiment has been completed Bennett and his group have reported that they found no change in the absorption rate of radioiodinated human serum from the intestinal tract of mice following sublethal radiation(5). In view of previous studies showing that the state of protein nutrition of an animal has considerable effect on the radiosensitivity of that animal(6), it seemed advisable to determine if parenteral administration of protein hydrolysate could produce more effective protein utilization than oral feeding following total body x-ray. For this reason the following experiment was undertaken in which groups of irradiated rats were given equal quantities of a protein hydrolysate (Amigen) orally and parenterally.

Procedure. Protein-depleted rats were used in this study since they are more sensitive to radiation than animals that have adequate protein reserves(6) and they have been shown to be a good test animal for assaying protein utilization(7,8). Ninety Sprague-Dawley male rats with an initial weight of 200 g were depleted of their protein reserves according to the procedure developed by Cannon and his group(9,10). When the animals had lost about 25% of their body weight after being maintained on the low protein diet for 10 weeks, they were random distributed into 3 series of animals of 30 rats each. Each series was then divided into 5 groups of 6 rats each and subjected to total body irradiation with the dosage ranging from 450 to 650 r. Following radiation all animals were housed in individual cages. All of the animals were weighed at 2-day intervals through the 30-day observation period. The enzymatic hydrolysate of casein, Amigen, was used in this study since the preparation was available both in powdered form for oral feeding and in 10% solution for parenteral administration. Previous studies have shown that Amigen promotes weight recovery in the protein-depleted rat comparable to that produced by the feeding of high quality protein(11). All of the animals in all groups received approximately

TABLE I. 6 Rats in Each Series. Diet: 3E + Amigen.

	Dose	Deaths
No inj.	450 r	0
	500	1
	550	3
	600	5
	650	6
Saline inj. 6 cc t.i.d.	450	0
	500	1
	550	3
	600	5
	650	5
Diet: 3E, 10% Amigen inj. 6 cc t.i.d.	450	2
	500	1
	550	3
	600	5
	650	6

1.8 g daily which is equivalent to 1.35 g of protein per day (NX 6.25). In all animals a basal low protein diet (3E) was fed to supply the caloric and vitamin requirements(9) and this diet was supplemented by the Amigen. The first series of rats received the basal ration (3E), to which 128 g of powdered Amigen had been added per kg, at the rate of 15 g per rat per day. No injections were given in these animals. The second series of animals was also fed 15 g per rat per day of the basal diet to which powdered Amigen had been added at the rate of 128 g per kg. All of the animals in the second series were injected with 6 cc of saline subcutaneously 3 times a day to serve as controls for the fluid intake of the third series. The third series was fed the low protein diet at the rate of 15 g per rat per day. These animals were given 6 cc of 10% Amigen subcutaneously 3 times a day.

Results. The weight changes for all the animals given 500 r of radiation are shown in Fig. 1. It is noted that the animals given Amigen subcutaneously did not undergo the weight loss during the first 3 or 4 days shown by the 2 groups fed orally. Weight loss during early radiation recovery is the usual finding. There was a somewhat greater rate of weight gain between the 3rd and 12th days in those animals not subjected to the trauma of injection.

The mortality results in this experiment are summarized in Table I. Mortalities were almost identical in all 3 series with the exception of 2 deaths at 450 r in the series given Amigen subcutaneously. Calculation of mortality regression lines, according to the method reported by Bliss(12), showed very little difference in the LD50/30 for the animals in the 3 series. The LD50/30 for the animals given Amigen subcutaneously was about 544 r, for the animals given saline subcutaneously about 554 r, and for those given no injections about 547 r.

Discussion. The weight curves show that there is no essential difference in weight recovery in the protein-depleted rat whether that animal is given protein hydrolysate orally or parenterally. The fact that the weight curve for those animals given Amigen subcutaneously rises almost steadily from the start of the administration of Amigen suggests that possibly intestinal absorption was impaired for the first 3 or 4 days in the other groups. It is doubted that this early weight increase is due to water retention for a similar rise is not seen in the series injected with saline. The weight recovery curves suggest that protein utilization is similar in the protein-depleted irradiated rat whether the hydrolysate is given orally or parenterally.

There is a slight divergence of the weight curves between the 6th and 12th days for the animals given no injection and those injected with saline. No reason can be offered for this divergence but it is noted that the curves are parallel for the last half of the experiment. For this reason it is felt that the trauma of multiple injections and the forcing of large volumes of subcutaneous fluid into the animal had very little, if any, deleterious effect on the animal. Autopsy of the injected rats at the conclusion of the experiment showed no edema, fibrosis, or infection in the subcutaneous area of the back despite the fact that the animals had been subjected to 90 subcutaneous injections and 540 cc of fluid.

The mortality regression lines indicate that there is no difference between feeding a protein hydrolysate orally or parenterally following radiation in the protein-depleted animal. The lines also indicate that multiple injections under sterile conditions do not have an adverse effect on survival in the radiated rat.

While this experiment shows that parenteral feeding of protein hydrolysate during radia-

tion recovery offers no advantage over oral feeding as measured by long-term weight gain or mortality, it does suggest that parenteral feeding may be advantageous during the first few post-irradiation days. For this reason further work with parenteral hydrolysate administration during the early post-irradiation period is indicated. In view of the earlier work of Buchwald reporting impaired intestinal glucose absorption in the immediate post-irradiation period(3), the effect of complete parenteral feeding in the early period following radiation should be studied.

Conclusions. 1. Parenteral feeding of protein hydrolysate following radiation offers no advantage over oral feeding in the protein-depleted rat as measured by radiation susceptibility. 2. Parenteral hydrolysate administration may be advantageous in preventing early post-irradiation weight loss.

The animals were irradiated through the kind cooperation of the Biology Division, Argonne National Laboratory, Chicago, Ill.

The author wishes to express his appreciation for the aid and assistance of the following: Paul R. Cannon, Robert W. Wissler, and Randolph H. Hughes, Department of Pathology, University of Chicago and Austin M. Brues, Biology Division, Argonne National Laboratory, Chicago, Ill.

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Received January 31, 1952. P.S.E.B.M., 1952, v80.

Inhibition of Growth of a Green Flagellate by the Antihistamine, β-dimethylaminoethyl (Benadryl).* (19504)

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The antihistamines and related drugs have been shown to have a variety of effects upon living systems, including growth inhibition of micro-organisms(1-5), inhibition of glutamate oxidation by rat brain tissue(6), and production of conduction block and depolarization of frog nerve(7). Of these effects, only one case of bacteriostatic activity(4) and one case of fungistatic activity(3) were reversible by histamine. Experiments in this laboratory have shown that β-dimethylaminoethyl benzhydryl ether (Benadryl hydrochloride, Parke, Davis Company) is a potent inhibitor of the growth of the green flagellate, *Chlorogonium tetragamum.*

Experiments were carried out in a modification of the medium of Hutner *et al.*(8) for fresh-water chlamydomonads, produced by reducing the concentrations of trace metals and chelating agent to one-fifth those described for the original medium. Dilution of this medium by one-sixth its volume, changes in initial pH from 5.5 to 8.0, and the addition of the metabolites and biological

* Aided by a grant from the California Cancer Institute.

materials mentioned below had no appreciable effect on the growth of *Chlorogonium* in this medium. The organisms were grown in 14 x 125 mm cotton-stoppered test tubes, and incubated at 25°C with constant fluorescent illumination. Populations were estimated by Tyndall-beam nephelometry† at approximately 3-hour intervals during the logarithmic growth period (about 12 hours) and at later times when necessary. Cultures were routinely plated on bacteriological media at the end of each experiment, and data from contaminated tubes were discarded.

Results. Benadryl‡ in concentrations of 1.7×10^{-5} M to 8.6×10^{-4} M causes a graded inhibition of the growth rate of *Chlorogonium* from almost normal growth at the lower concentration to almost complete inhibition at the higher. Concentrations in this range are not lethal, since subcultures into Benadryl-free medium after 24 hours exposure to Benadryl regularly showed normal growth. Cells maintained in 8.6×10^{-4} M Benadryl were normal in appearance, motility, and phototropic behavior after 24 hours. Higher concentrations of Benadryl, of the order of 10^{-3} M, caused actual death of the cells within a few hours.

The growth inhibitory effect of Benadryl is extremely sensitive to pH, as its action in causing nerve block(7). No inhibition is produced at pH values below ca. 6.8. Since the pH of growing *Chlorogonium* cultures rises continuously, cultures containing Benadryl and having an initial pH below this critical level grow normally for a period of time inversely related to the initial pH, after which time they show marked inhibition (Fig. 1). This dependence on pH seemingly indicates that only the free-base form of Benadryl is inhibitory. Whether this is due to differences in the pharmacological activities or in the permeability of the cell membrane to the two forms is not clear. Since many of the materials added in the experiments described below tended to acidify and/

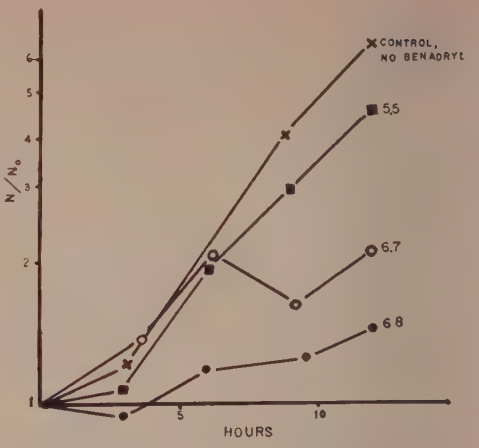

FIG. 1. Relation of Benadryl inhibition to pH. Benadryl concentration (except in control), 8.6×10^{-4} M. Points represent averages of 3 replicate tubes. Figures at ends of curves indicate initial pH. N equals population at time indicated on abscissa; N_0 equals initial population.

or buffer the medium, great care was required to prevent pH effects from interfering with the results.

Attempts were made to reverse the Benadryl inhibition by addition of various substances to the medium. Histamine in a molar ratio to Benadryl of 8:1 had no effect. Thiamin and nicotinamide in combination have been reported to reverse the inhibition of bacterial growth by another antihistamine(1), but these compounds, in molar ratios to Benadryl of 11 and 14 respectively, had no effect on the inhibition, whether added alone or in combination. The inhibition of glutamate oxidation in brain(6) and accumulation of pyruvic acid in bacterial cultures(4) caused by antihistamines suggested that these compounds might affect the Krebs cycle, but neither malic nor α-ketoglutaric acid (molar ratios of 15) nor a mixture of lactic, pyruvic, malic, α-ketoglutaric, succinic, fumaric, and citric acids (molar ratios ca. 2.5) reversed the inhibition. Neither acid-hydrolysed casein (Nutritional Biochemicals Co., "vitamin-free") nor yeast extract (Difco) (in 1% concentrations) was effective.

In a few experiments the growth-inhibitory activity of 4,4-diphenyl-N,N-dimethylbutyl-1-

† Unpublished technic developed by Roger W. Hanson.

‡ This compound and several others were generously supplied by Dr. Frederick Crescitelli.

amine hydrochloride,§ closely related structurally to Benadryl, was tested. But this compound possessed about one-fifth the antihistaminic activity of Benadryl. This compound was found to inhibit growth in a manner similar to Benadryl, but appeared to be even more potent than Benadryl itself, causing death of a large proportion of the cells in 24 hours at a concentration of 4.6×10^{-4} M, and producing total inhibition of growth at 2.3×10^{-4} M.

The lack of activity of histamine in reversing the Benadryl inhibition of *Chlorogonium* growth, and the lack of correlation between antihistaminic activity and growth-inhibitory activity in structurally related compounds appear to indicate that the growth inhibition is not related to the antihistaminic properties of the drug. The effect of Benadryl on *Chlorogonium* appears to differ from the bacteriostatic effect of antihistamines reported by Krecek *et al.*(4) and from the fungistatic effect reported by Landis and Krop(3), which were reversed by histamine, and from the bacteriostatic effect reported by De Ritis and Zanussi(1), which was reversed by a thiamin-nicotinamide mixture. Reiss and Caroline(5) have reported fungistatic effects of antihistamines which were not reversed by histamine, nor did histamine reverse the effects of Benadryl on vertebrate nervous tissue(6,7). Thus, it seems likely that the antihistaminic drugs may produce their effects by several different mechanisms.

§ Synthesized by Dr. T. A. Geissman, Chemistry Department, U.C.L.A., and tested for antihistaminic activity (guinea pig aerosol technic) by Dr. E. J. Fellows, Smith, Kline and French Laboratories, Philadelphia.

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Received February 13, 1952. P.S.E.B.M., 1952, v80.

Effects of Acute Radiation on the Adult Mammalian Central Nervous System.* (19505)

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A study in progress in this laboratory has been directed to characterize the changing metabolism of the nervous system as it develops from embryo to adulthood and to relate this to its changing radiosensitivity. Data on neurectoderm, neuroblasts and neonatal nerve cells have been reported elsewhere(1-3). In general the adult nervous system is considered to be radio-resistant, but whether it is uniformly so has not been determined.

In order to learn whether a differential of radiosensitivity exists within the adult nervous system, rats and mice were irradiated either to the head or to the whole body and the acute histopathologic changes that followed were recorded. The radiation given ranged from 50 to 20,000 r.

Materials and methods. Adult rats and

* This work was done under U. S. A. E. C. Contracts, AT(30-1)901 and AT(30-1)699, U. S. P. H. S. Grant C-1042 and a Grant from the United Cerebral Palsy Associations.

mice of both sexes from 3 months to about one year old were given single exposures of x-rays, either total body, or to the head only with the body shielded by lead. Radiation intervals used were 50, 100, 200, 300, 400, 600, 800, 1200, 1500, 1600, 2400, 2800, 5000, 10000, 15000, and 20000 r. Both normal rats and mice and some mice bearing certain tumors were used. The tumor bearing animals were used because they offered additional material in the total body experiments, and there was no evidence that the tumors altered the radiation effects. Included in the study were additional multiple brain sections from animals previously reported as negative(4). Groups of 3 to 6 animals were used for each dose level of radiation. Rats only were used for the head experiments. X-ray factors were: General Electric Maximar therapy unit, 200 kv, 10 ma, inherent 3 mm aluminum filter, 25 cm target distance. Frequent checks were made with a Victoreen apparatus (in air). Animals were killed 6 to 48 hours after radiation except in the head experiments where the rats were in some instances allowed to survive for several days or until they died, which was usually on the 9th to 11th day. They received 1500 to 5000 r. Complete autopsies were performed, and sections made of all organs in about half of the animals, and of lymphoid tissue, spleen, muscle, bone marrow, small intestine, gonads, and nervous system only in the remainder. The nervous system was examined by multiple sections of the brain, sections of the gasserian ganglia, 2 levels of the spine including cord and associated ganglia (thoracic and lumbar), and sciatic nerve. In some of the 15,000 and 20,000 animals the thoracolumbar sympathetic chain was excised. A number of skeletal muscles were sampled in most cases. Fixation was in Bouin's or Zenker's fluids, embedding in paraffin and staining by hematoxylin and eosin usually, but also eosin-methylene-blue, Bodian protargol, phosphotungstic hematoxylin and iron hematoxylin for myelin. Comparisons were made with adult, newborn and fetal animals that were irradiated, or treated with radiomimetic drugs, sulfhydryl reagents or other forms of metabolic inhibition(1-3). Changes in the intestine, testes and hemopoietic organs served as a rough biological check on radiation.

Results. X-rays produced necrosis of several different groups of cells in the central nervous system. Those affected were oligodendroglia, neurons in parts of the olfactory brain, subependymal cells adjacent to the ventricles, especially the lateral, and rod cells of the retina.

Necrosis of retinal rod cells was seen in the 5 rats killed between 6 and 11 days but did not appear before 6 days. It was not observed in any of the animals with total body radiation (killed within 48 hours). Necrotic rod cells were seen in varying stages of disintegration, although the whole layer of these cells was sometimes destroyed, leaving only scant residual eosinophilic debris. (This strain of rats did not show congenital absence of rod cells.) In younger animals with head or total body radiation the small subependymal cells of the lateral ventricles were necrotic within 6 to 12 hours. When present in relation to other ventricles they were also affected. Necrosis was always considerable at 200 r but in both rats and mice necrotic cells were rare or absent at 50 and 100 r. These subependymal cells are small with scant cytoplasm, a compact deeply basophilic nucleus and they are indistinguishable from the immature subependymal and neuroblastic cells of this region in embryos and newborn animals(3).

In all animals with total body radiation or radiation to the head above 1200 r necrotic oligodendroglia cells were present within 6 to 24 hours after radiation. These were scattered widely in the white and gray matter but were not numerous. In a number of animals with total body or head irradiation above 1200 r there was occasional necrosis of nerve cells in the granular layers of the olfactory bulb and of larger cortical neurons in the pyramidal lobe. In no animals were necrotic cerebellar granule cells seen as sometimes happens after radiomimetic drugs. Dead oligodendroglia and nerve cells were characterized by marked reduction in cell size, poorly outlined eosinophilic cytoplasm and a strongly basophilic but blurred nucleus markedly reduced in size. Identification of necrotic oligodendroglia cells was made by seeing them,

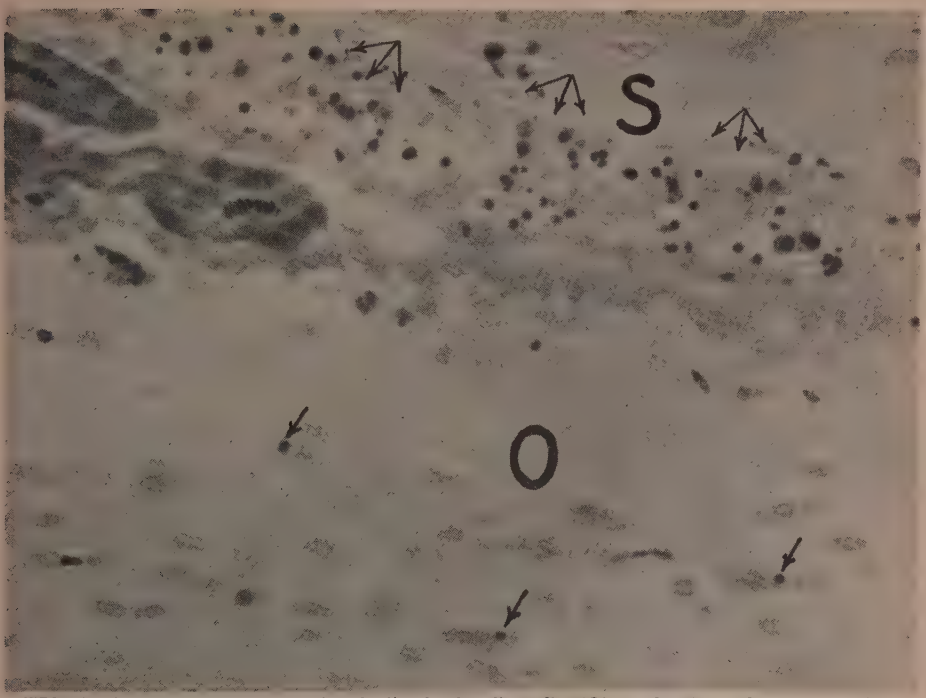

FIG. 1. Acute radiation necrosis of oligodendroglia cells (O), and subependymal cells (S). Brain of a young adult rat 6 hr after 1500 r total body irradiation. Three dead oligodendroglia cells in the corpus callosum are indicated by arrows above, and numerous necrotic subependymal cells are evident below by single or clumped masses of chromatin and indistinct cytoplasm. (Eosin-methylene blue. × 500.)

usually singly, in a row of oligodendroglia cells in such areas as the corpus callosum (Fig. 1). Necrotic satellite cells around neurons in the gray matter were presumed to be oligodendroglia by their size and shape, but in this state of destruction distinction from microglia cells was not certain. Bodian protargol and myelin stains revealed no change in the axis cylinders or myelin adjacent to regions where acutely necrotic oligodendroglia cells were observed.

No significant changes were seen in the sympathetic ganglia. Occasional vacuoles in sympathetic neuron cytoplasm and irregular cell staining occurred but these were also observed in control material. The spinal cords were negative except for occasional necrotic oligodendroglia cells. Peripheral nerves (sciatic) were negative. Rarely was damage to skeletal muscle seen, although samples were made of diaphragmatic, abdominal, intercostal, masseter, neck organs, spinal, thigh, extrinsic eye and external ear muscles. In the diaphragms of mice and rats given 15,000 r and killed 24 hours later, necrosis with disintegration of occasional muscle fibers occurred. Bone marrow, lymphoid tissue, gut, salivary glands, testes and ovaries showed characteristic acute radiation changes depending on the dosage and interval after radiation. Changes in gut, lymph nodes, testes were evident in 24 hours at 200 r, and absent or equivocal at 100 r. Thus they were of a radiosensitivity comparable to that of subependymal cells but the variables inherent in these experiments do not justify a closer comparison.

Summary. Irradiation of rats and mice resulted in acute necrosis of scattered oligodendroglia cells, subependymal cells when present in younger adult animals, retinal rod cells, and occasional neurons in the pyramidal lobe and olfactory brain. Subependymal cells were

radiosensitive (200 r) but the other cells were not destroyed below 1200 r.

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Received March 5, 1952. P.S.E.B.M., 1952, v80.

Comparative Uptake of BR^{82} by the Hypophysis and Other Tissues.* (19506)

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Bernhardt and Ucko(1), and Zondek and Bier(2) reported that bromide was a normal constituent of the blood, that it could be detected in a variety of tissues, and that the content of the hypophysis was strikingly high. In both man and the dog hypophyseal tissue gave values of 5 to 30 mg of bromide per 100 g of wet tissue as compared to 0.5 to 1.5 mg % for the blood and most other tissues. More recently Dixon(3) and Ucko(4), using newer analytical methods, failed to confirm the existence of any preferential concentration of bromide in the hypophysis of either the ox, pig, or man. Perlman, Morton, and Chaikoff(5) employing Br^{82} could not demonstrate a high concentration of radiobromide in pooled samples of pituitary tissue from a group of 8 rats.

A selective collection of bromide by the pituitary gland would be of considerable interest because of the implication that bromine might participate in the synthesis or discharge of pituitary hormones. Moreover, a therapeutic application of radiobromine for localized radiation of the gland would be possible if the collection of the isotope greatly exceeded that attained by other tissues. In the present study a determination was made of the uptake of Br^{82} by the hypophysis and by other organs of the rat and rabbit. Any uncertainty as to the specificity or reliability of a chemical method is avoided by the use of the radioactive isotope.

Methods. Br^{82} obtained from Oak Ridge National Laboratories in the form of irradiated KBr was assayed by comparison with a Radium D plus E standard obtained from the National Bureau of Standards. Correction for the difference between the energy of beta particles of the standard and Br^{82} was accomplished by extrapolation to zero-absorption from counts obtained with a series of aluminum filters. Rate of decay was measured in order to verify the absence of appreciable quantities of K^{42} which, although present in fresh samples of KBr immediately after irradiation in the pile, rapidly disappears from the mixture because of its relatively short half-life.

Eleven rats and 12 rabbits were given Br^{82} in a dose of 0.5 to 1.0 millicurie per kilo by slow intravenous injection. The amount of KBr received averaged 28 mg/kilo and ranged from 18 to 45 mg/kilo. After 24 hours the animals were anesthetized with ether and exsanguinated by incision of the dorsal aorta. Tissue samples were weighed in the wet state. With small organs such as the pituitary, thyroid, adrenals, and ovaries, the whole gland was digested with 10% KOH and evaporated to dryness in a one-inch steel counting cup. Beta counts were obtained with a mica window Geiger counter and corrections made for self-absorption. When, as with larger organs, a sample weighing more than 0.1 g could be obtained, the activity was measured by gamma counting. This was accomplished by means

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

TABLE I. Tissue Content of Br^{82} at 24 Hr. The meaning of concentration ratio is explained in the text.

Tissue	Concentration ratio and stand. error of mean		Tissue-plasma ratio	
	11 rats	12 rabbits	11 rats	12 rabbits
Serum or plasma	3.64 ± .05	3.18 ± .12	1.00	1.00
Whole blood	2.78 ± .03*	2.30 ± .01	.74*	.83
R.B.C.'s	1.62 ± .03*	1.82	.44*	.65
Stomach contents	—	3.67 ± .43*	—	1.01*
Stomach	2.84 ± .30	2.10 ± .24	.79	.70
Kidney	1.66 ± .30	1.51 ± .10	.46	.48
Spleen	1.55 ± .19	1.22 ± .08	.43	.39
Liver	.80 ± .08	.82 ± .08	.22	.26
Gonad: Testis	1.87 ± .03	—	.52	—
Ovary	—	1.17 ± .18	—	.37
Adrenal	.84 ± .08	.65 ± .03	.21	.21
Small intestine	1.25 ± .40*	.90 ± .02*	.36*	.25*
Large "	.48 ± .10*	—	.14*	—
Brain	.47 ± .02*	.68 ± .01	.13*	.22
Muscle	.44 ± .05	.38 ± .04	.12	.13
Thyroid gland	1.91 ± .18	.80 ± .03	.49	.26
Pituitary gland†	1.34 ± .15	.84 ± .08	.36	.28

* Performed on half of total number of animals.
† No attempt was made to separate the anterior and posterior lobes.

of an ionization chamber fitted with a well into which a test tube could be introduced, and which was connected to a Vibrating Reed Electrometer and Brown Recorder. The tissues were counted without digestion. It was established that variations in the shape or size of a sample (between 0.1 and 2 g) had no significant effect on the magnitude of a count. Standards obtained from the original solution used for preparation of the administered doses were counted along with each series of tissue samples. Physical decay of the Br^{82} was thereby corrected automatically. The content of Br^{82} was calculated as a ratio of the activity per g of tissue of an organ divided by the activity of the dose administered per g of animal.

Results and discussion. The activity of Br^{82} found in various organs after a 24-hour interval is presented in Table I. The ratio of activity of tissue to that of plasma in the final 2 columns allows comparison with similar ratios previously obtained by chemical methods. Bromide, administered in pharmacological doses, moves into the interstitial fluid in equilibrium with plasma in the same way as chloride, except in brain and spinal fluid where Br/Cl ratios are lower than in other organs(6,7). In the event that the tissue/plasma ratio of Br^{82} should exceed unity, conclusive evidence would be at hand that the cells of the organ are able to concentrate bromide ion. If the cells do not take up this ion the activity of tissue would vary with the content of interstitial fluid and the small component of retained blood. In order to detect a small increment of intracellular Br^{82} the extracellular fluid volume of the sample would have to be measured. The method here employed is applicable only for the detection of gross increases in cellular concentration.

As would be expected, the highest concentration in all groups of animals was in plasma. Red cells contained approximately half as much as plasma. The relatively high values for stomach contents and stomach tissue may be explained on the basis of partial substitution of bromide for chloride in gastric juice. I^{131} has been shown to behave similarly(8). The hypophysis did not contain a consistently higher concentration of Br^{82} than other glandular organs nor was the thyroid remarkably high in activity.

The above findings are in accord with the chemical determinations of Dixon(3) who did not find an unusually high content of bromide in the pituitary and who moreover demonstrated that the Br/Cl ratio of this organ was similar to that of a variety of other tissues in both man and several other experimental

animals. His tissue-serum ratio of bromide in the pituitary of the pig was 0.22. This compares reasonably well with the ratio of 0.28 for rabbits and 0.36 for rats in the present series, and contrasts sharply with the ratio of 17 obtained by Bernard and Ucko(1).

The results are also in agreement with those of Perlman *et al.*(5), who found no selective uptake of Br^{82} by the hypophysis of the rat. At variance is the failure to demonstrate preferential uptake by thyroid tissue. Perlman and coworkers found a higher concentration of Br^{82} in the thyroid gland than in plasma, and also succeeded in augmenting the content of the gland by 30% with thyrotropin. The lack of such selective concentration of Br^{82} in the animals of the present series might be dependent on either a fortuitously low level of thyroid activity in the animals, or the existence of a state of relative iodine saturation. It would appear from the work of Bauman, Sprinson, and Marine(9) that a significant substitution of bromine for iodine requires the presence of a pre-existing iodine depletion. In a recent report by Verkhovskaya(10) the Br^{82} uptake of the pituitary, while in most instances not different from that of other organs, was in 2 out of 19 rats, 15 to 30 times greater than in the remainder of the animals. Although it is possible that a variation in the functional status of the gland might be responsible for such divergent values, a technical error would seem to be a more likely explanation. Dixon found no more bromide in the pituitary of the ox, in which gonadotropin synthesis should be very pronounced, than in the cow or bull.

Summary. In confirmation of previous work(3,5), no evidence was adduced for the selective concentration of bromide in the form of Br^{82} by the pituitary gland of the rat and rabbit. The activity of gastric contents and gastric tissues was relatively high, but no preferential uptake by thyroid tissue(5) was demonstrated.

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Received March 7, 1952. P.S.E.B.M., 1952, v80.

Some Results of Substituting Other Nerves for the Phrenic. (19507)

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The reports of Brown and Satinsky(1,2) on successful substitution of the phrenic nerve by the vagus caudal to its inferior laryngeal branch suggested the possibility of using other nerves for the same purpose. The results of a variety of such cross anastomoses are reported in this paper. In general, powerful contractions of the diaphragm are obtained on direct stimulation of these nerves, but lack of proper central control makes them inadequate to serve as the only route for respiratory impulses. In this respect the results of the present study differ from those obtained by Brown and Satinsky after vago-phrenic anastomosis, and likewise from the results reported by Colledge and Ballance(3) following cross anastomosis of the descendens hypoglossi and the phrenic nerve.

Materials and methods. Under nembutal anesthesia the trapezius portion of the acces-

sory nerve was anastomosed to the phrenic nerve on the right side of 5 adult cats and the long thoracic nerve was used likewise in 2 cats and 5 rats. In one cat buccal branches of the right facial nerve were anastomosed to the distal end of the fourth cervical contribution to the phrenic. The nerve ends were held together with a single loop of .005 or .003 inch tantalum wire. The wire was sharpened under a binocular microscope and each nerve was stabbed through the middle one or two millimeters from its cut end. The wire was then formed into a small loop bringing the two cut ends in contact. The anastomosed nerves were under no tension as previous trials with tension nearly always resulted in separation of the opposed ends.

Results. After 140-218 days, the site of anastomosis was exposed and the nerves freed from surrounding structures. The substituted nerve was then stimulated with a Harvard inductorium 2-3 cm above the site of anastomosis. Vigorous contraction of the diaphragm occurred in all cats tested; of these, 4 had an accessory, 2 had a long thoracic, and one a facial nerve substitution. The 2 surviving rats with long thoracic replacement gave similar results. Procedure from this point varied with developing experience. In the cat with the facial anastomosis, the opposite phrenic, both long thoracic nerves and the infrahyoid muscles were cut. These procedures resulted in a considerable increase in respiratory effort. The right facial nerve then was cut and additional respiratory effort followed, but the cat continued to breathe regularly for 20 minutes presumably with the intercostal muscles only. The animal was then sacrificed. After death, stimulation of the distal end of the cut facial nerve caused vigorous contraction of the exposed diaphragm. In the other animals the adequacy of the substituted nerve was checked by transection of the cord between the eighth cervical and first thoracic vertebrae combined with avulsion of the left phrenic nerve. Only one animal breathed rhythmically after these maneuvers, using the right side of the diaphragm. However, it continued this respiration after the substituted nerve was cut. Further dissection of the phrenic nerve at the thoracic inlet led to abrupt cessation of all diaphragmatic movement in this animal indicating the presence of accessory phrenic fibers that escaped notice at the time of anastomosis. Failure of respiration in these animals was not due to insufficient motor innervation of the diaphragm by the substituted nerve as demonstrated by the following procedure. After cord transection and left phrenic avulsion 2 cats and one of the rats were kept alive for an hour by periodic electrical stimulation of the substituted nerve.

Discussion. Brown and Satinsky consider x-ray visualization of diaphragmatic movement and severance of the opposite phrenic nerve as sufficient proof of the effectiveness of vago-phrenic anastomoses. Colledge and Ballance depended on x-ray visualization alone. Such measures are inadequate in the rat and cat since they survive very well, at least for short periods, on the intercostals alone. In these species, elimination of all intercostals, the other phrenic nerve, and careful exclusion of any remaining phrenic fibers on the operated side are necessary for the evaluation of a phrenic nerve substitution in acute experiments. When these precautions are taken, the facial, the accessory and the long thoracic are unable to induce rhythmic contractions when innervating the diaphragm. This failure is not due to diaphragmatic weakness but to the absence of rhythmic bursts of nerve impulses even under extreme respiratory distress. The failure of the "external respiratory nerve of Bell" suggests that this term for the long thoracic is a misnomer at least when applied to the cat and rat. The motor adequacy of the nerve crosses suggests that a human being with the same types of anastomosis could breathe with such an arrangement by attempting to move the muscle ordinarily supplied by the nerve anastomosed to the distal end of the phrenic. In other words, while awake he might substitute dependence on a respiratory machine for voluntary efforts that would result in adequate contractions of the diaphragm.

Summary and conclusions. The spinal accessory, the long thoracic and facial nerves were substituted for one phrenic nerve in cats and rats. All of these nerves will supply motor fibers to the diaphragm sufficient for it

to serve as the only respiratory muscle. Lack of proper central connections prevents spontaneous use of this power in the animals tested.

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Received March 7, 1952. P.S.E.B.M., 1952, v80.

A Simple Electrode Carrier for the Exploration of Subcortical Structures.* (19508)

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Electrode carriers for use in the stimulation of or recording from subcortical areas have been developed by Hess(1), and his school (2), Harris(3), Hunter and Jasper(4), and recently by Knowles(5). These technics for implanting brain electrodes, particularly that developed by Knowles, are elaborate and laborious, requiring careful construction of various parts and sub-assemblies, such as base plates, electrode carrier plates, assembly jigs, and electrode carrier assemblies. In addition, the curvature of the skull necessitates, in the Knowles method, the formation of a broad foundation to receive the electrode carrier or base plate; and finally the electrode carrier must be fixed securely and aligned with great exactness in the plane of the stereotaxic apparatus, a process demanding repeated checking and leveling of the electrode carrier. All this is avoided in the method described here, yet the electrodes are accurately placed, the procedure is simple and quick, and no assemblies or sub-assemblies are used.

Procedures. Under general anesthesia a piece of 3/8 inch plexiglas, 15 mm x 20 mm (a, Fig. 1) is fastened over a trephine opening in the skull as securely as possible, disregarding its orientation to the stereotaxic apparatus in which the animal is fixed. A dental drill mounted in the electrode holder of the stereotaxic apparatus is then used to drill guide holes (b, Fig. 1) at the chosen coordinates and in the desired direction. Upon recovery from anesthesia, the electrodes (c, Fig. 1) can be inserted without discomfort to the animal to the desired depth. The depth may be varied throughout the experiment, or the electrode

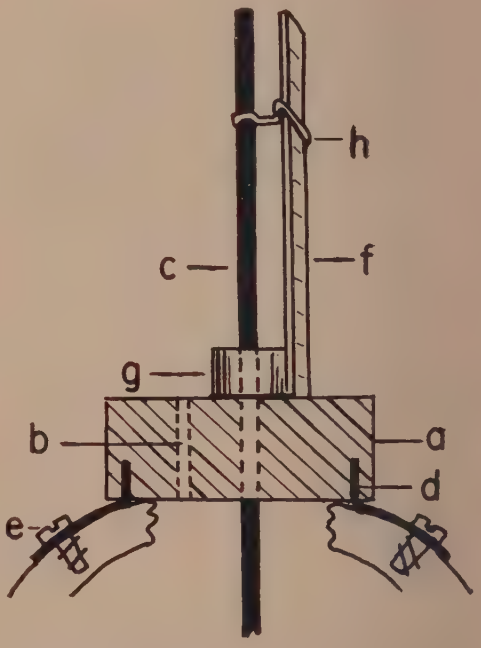

FIG. 1. Plexiglas plate with electrode in place. a—plexiglas plate; b—guide holes; c—electrode; d—solder lug; e—screw; f—scale; g—stop; h—scale indicator; i—spring.

* Aided by a grant from the National Institute of Mental Health of the National Institutes of Health, U.S.P.H.S.

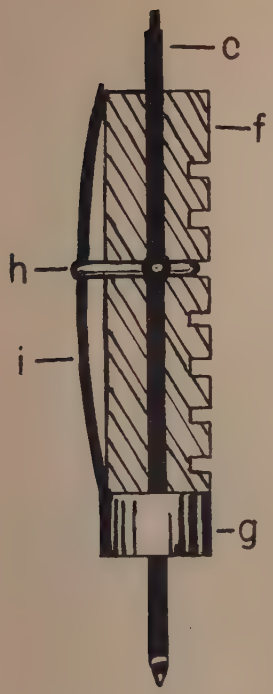

FIG. 2. Electrode with scale and stop.

may be cemented in place at any desired depth. Thus the electrodes are oriented with reference to the stereotaxic coordinates but the plate carrying them is not. This prevents errors which might be introduced by slight disorientation of the carrier plate such as occurs in the methods previously cited.

The plate itself is attached to the skull of the anesthetized animal by a pair of small, doughnut type solder lugs (d, Fig. 1) which are heat crimped, one on each side, into the bottom of the plexiglas. This is placed on the skull in a secure position, and the flexible lugs are bent to follow the curvature of the bone. The opening of each lug is marked on the bone and holes for the fastening screws (e, Fig. 1) are drilled with a dental burr. Self-tapping sheet metal screws fix the plate rigidly. The electrode holder carries a dental drill which is fitted with a bit of desired size whose shank had been adapted to fit the chuck of the drill. The diameter of the bit should insure a snug fit of the electrode in the guide hole. The transparent plexiglas permits drilling of the guide holes without injury to the brain, and the desired depths for the electrodes are easily reckoned since the distance between the cortex and the top of the plate can be seen and measured. The guide holes can be placed within a millimeter of each other without difficulty.

In addition to the plexiglas plate, the apparatus consists of one or more bipolar, concentric needle electrodes, insulated to the tip, and a scale (f, Fig. 1 and 2) notched at one or 2 mm intervals, and cemented at its base to a small cylindrical stop (g, Fig. 1 and 2). The stop is made of hard rubber, center drilled to allow free movement of the electrode, and has one side filed flat to accept the millimeter scale. The scale indicates the position of the electrode in the vertical plane. The scale indicator (h, Fig. 1 and 2) is a flat loop of wire fastened to the upper portion of the electrode. The indicator fits into a notch at the desired scale reading and is held at the chosen level by a spring (i, Fig. 2) which is a bow of piano wire cemented to the base of the scale. Upward movement of the stop-electrode combination is prevented by a small rubber band slipped under the solder lugs just prior to fastening the plate to the skull, and brought twisted over the top of the plexiglas where it can be set to hold the stop flush against the plate.

With the plate fastened to the skull of the anesthetized animal and the guide holes drilled, the incision is closed with a purse string suture. A strip of gauze is then laid around the plate and sealed over the wound with collodion. The animals seem in no way discomforted by the presence of the plexiglas plate.

This method has been used both for stimulating the midbrain, thalamus, and hypothalamus, and for recording potentials from these areas. Histological examination has established the accuracy of electrode placement.

Summary. A simple, inexpensive and quickly executed method for exploring subcortical areas is described.

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Received March 17, 1952. P.S.E.B.M., 1952, v80.

Intracellular Development of Vaccinia Virus.* (19509)

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The cytoplasmic inclusions formed in cells infected with vaccinia virus have been recognized for 60 years and extensively studied with the optical microscope. It is generally accepted that the inclusions consist of masses of elementary bodies, but the manner in which they are formed is still uncertain and several conflicting views are held by various authors. The literature on this subject has been reviewed by Van Rooyen and Rhodes(1).

Recent technics of making ultra-thin tissue sections(2-5) have been utilized by Bang(6) and Wyckoff(7), who have each published electron micrographs made from thin sections of infected chorioallantoic membrane. They clearly show the inclusions to be aggregates of elementary bodies. The development of the inclusion bodies has not been reported, however, and it is with this phase of the problem that the present paper is concerned.

Methods. Ten to 12-day embryonated eggs were inoculated on the chorioallantoic membrane with approximately 10^8 infectious doses of virus in order to have as many cells infected as possible. The suspensions of virus were prepared and the inoculations were carried out according to the method of Briody and Stannard(8). The virus was a commercial calf lymph strain of vaccinia (Lederle) passed once in rabbit skin and 3 times in eggs. Membranes were harvested 48 hours after inoculation and fixed for 24 hours in 4% formalin buffered with M/10 phosphate at pH 7.0. The tissue was dehydrated in graded alcohols, starting at 10%, and imbedded in 1:8 methyl:butyl methacrylate. Polymerization was carried out at 60°C overnight using 2-4 dichlorobenzoyl peroxide as catalyst(3). Sectioning was performed on a Spencer microtome modified with a 1:20 wedge and driven by a motor as described by Hillier and Gettner(4). A glass knife(5) was used for cutting. The sections were lifted from the water with collodion covered grids and the methacrylate was removed from the sections with toluene before examination with an RCA electron microscope, Model EMU.

Results. In the ectodermal layer of 48-hour membranes nearly every cell examined was infected with virus and in any one group of cells, inclusions ranging from a few particles to those occupying most of the cytoplasm could be observed. In almost every cell containing a small or medium size inclusion, the inclusion was located at the nuclear membrane and consisted of particles of varying sizes enmeshed in a dense matrix (Fig. 1-5). The matrix appeared less dense in the larger inclusions. Recognizable virus was never seen within the nucleus.

Occasionally, cells were seen to be ringed with virus particles which filled the intercellular spaces (Fig. 5). Cells in such areas contained little and, occasionally, no detectable virus. In other areas, cells appeared to have

* Aided by a grant from the Fluid Research and James Hudson Brown Memorial Funds of the Yale University School of Medicine.

The data for this paper form part of a dissertation to be presented by William H. Gaylord, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University.

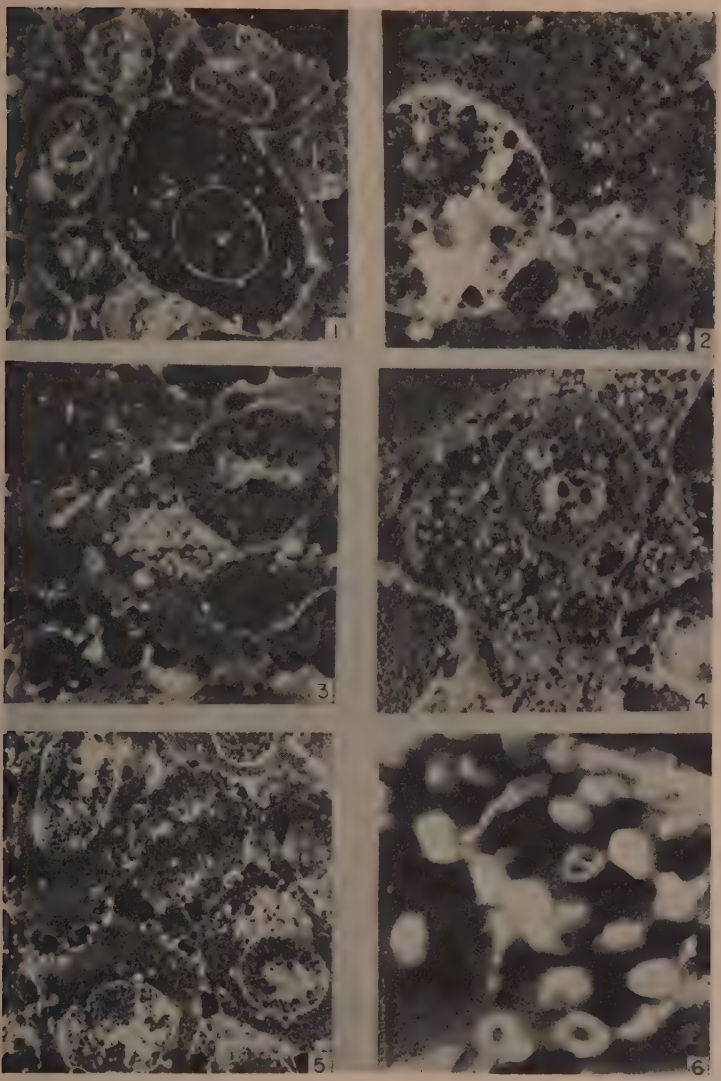

FIG. 1. Ectodermal cells of the chorio-allantoic membrane infected with vaccinia virus. The large cell in the center has only a few particles of virus and is probably recently infected. Other cells show varying stages of development of inclusion bodies. × 2300.

FIG. 2. A small inclusion showing definite attachment to nucleus. × 6000.

FIG. 3. A fairly large inclusion. The surrounding matrix is less dense. Note mitochondria in both filamentous and swollen forms. × 4000.

FIG. 4. A cell filled with virus and apparently rupturing. Palladium shadowed. × 3000.

FIG. 5. General view of ectodermal cells most of which are surrounded by virus particles. This is possibly the result of the liberation of quantities of virus from neighboring cells. One cell to the right appears to be infected in the next cycle of virus growth, for a perinuclear inclusion is present. × 2300.

FIG. 6. Virus particles some of which have been sliced by the knife. Note dense wall of two particles and apparent internal structure in one. Nuclear membrane at upper right. Palladium shadowed. × 30000.

several isolated virus particles in the cytoplasm but no real inclusion bodies. (See large cell of Fig. 1). Multiple inclusions were seen infrequently, with the majority of cells containing only one.

The general appearance of the infected cells gave the impression that no obvious cytopathologic changes had taken place. Occasional swollen cells, as in Fig. 1, were rare and the vacuolation shown at the border of the same group of cells could be seen in normal tissue. Although the cells seen in Fig. 5 pulled away from each other leaving large intercellular spaces, the effect could not be ascribed to the infectious process *per se* because other infected areas had closely packed cells. Few recognizable mitochondria have been observed. The nucleoli of normal and infected cells varied considerably in size and shape but a role in infection was not apparent.

In tissue sections, the elementary bodies of vaccinia appeared spherical to ovoid but never brick-shaped with sharp angles (Fig. 6). In any one cell, particle size varied, ranging from 0.23 μ to nearly 0.28 μ in length. Many torelike or doughnut shapes were seen and were interpreted as particles which had been twice sliced by the knife removing segments from both sides. Occasionally dense material was observed within such structures (Fig. 6). Central protuberances appeared on some particles suggesting the presence of a central mass similar to those found by Dawson and McFarlane after treating elementary bodies with pepsin(9).

Discussion. It is generally assumed that viruses are adsorbed to cells, penetrate the cell wall, multiply, and the new generations are released to infect other cells. The pictures presented here would support this theory. In Fig. 1 only a few particles are seen scattered through the cytoplasm. In Fig. 2 and 3, there are single inclusions of different sizes. Fig. 4 shows a cell filled with virus and apparently rupturing. In Fig. 5 the large amount of extracellular virus in an area in which cells contain only early inclusions, suggests an early phase of a second round of infection. It is possible to observe all phases of the development of the inclusion in a late lesion because vaccinia infection induces marked ectodermal proliferation and as new ectodermal cells are formed they become exposed to virus liberated from older cells.

The constant finding of inclusions in contact with the nuclear membrane suggests that their formation is in some way influenced by the nucleus. Ewing(10) concluded from his study of sloughed cells of vaccinia-infected rabbit cornea that the inclusion was continuous with the nucleus and was a result of the outward diffusion of nuclear material. Goodpasture, Woodruff, and Buddingh(11) emphasized the perinuclear position of inclusions in their classical work demonstrating that the inclusions consist of masses of elementary bodies.

The fact that the matrix in which the virus particles were imbedded was dense in small inclusions and almost absent in larger ones may be significant. If the nucleus is supplying nucleic acids, it might be expected that the loss of matrix material could be correlated with staining reactions. Several authors (12,13) have noted that the reactions of inclusions change with age from basophilia to eosinophilia and that there is also a progressive diminution in the intensity of the Feulgen reaction.

Bang(6) has suggested that there might be a relationship between mitochondria and vaccinia virus. Few mitochondria were seen in the present study (Fig. 1, 3, 4) but filamentous, swollen, and club-shaped intermediates occurred and no significant association with virus was noted. The plaque-like areas described by Wyckoff(7) in chorioallantoic cells infected with vaccinia were never seen, although somewhat similar areas have recently been described by Banfield *et al.*(14) in *Molluscum contagiosum.* Occasionally, particles about the size of mature virus could be seen in normal cells but virus could be clearly distinguished because of its greater electron density and uniformity of size and shape.

Summary. 1. Electron micrographs of tissue sections of chorioallantoic membrane infected with vaccinia virus were examined. Some cells were found to be almost filled with virus, others contained the virus particles grouped in single inclusions of varying sizes. Only a few virus particles were seen scattered

throughout the cytoplasm of cells in certain areas, and in other areas extra-cellular virus was found in large numbers. 2. Small or medium sized inclusions were always located at the nuclear membrane and in contact with it. The inclusions consisted of masses of virus particles imbedded in a matrix which was dense in small inclusions and less dense in larger inclusions. The virus particles were ovoid and varied in length from 0.23 μ to 0.28 μ. Sectioned virus particles indicate that all have a dense outer wall, and some could be observed to have an internal structure. 3. The possible relationship of these observations to the growth cycle of vaccinia virus has been considered.

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Received March 24, 1952. P.S.E.B.M., 1952, v80.

Concentration of Influenza Virus (PR8 Strain) by a Cation Exchange Resin.* (19510)

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The application of ion exchange resins to the separation of closely related chemical substances by adsorption or exchange of ions has proven in the past few years to be a valuable adjunct in the purification and concentration of biological products. Ion exchange resins have been used previously to purify partially a number of neurotropic viruses(1). The resins in this case were so adjusted that when suspensions of infected chick embryos and mouse brains were passed through columns of resin, inert nitrogenous material was removed and the virus appeared in the percolate fluid. Poliomyelitis and Theiler viruses have also been partially purified by adsorption of the viruses and inert nitrogenous material on anion exchange resins followed by selective elution of the viruses(2).

In the present experiments a method has been developed to concentrate rapidly and to purify partially the PR8 strain of influenza virus. It was observed that the virus was selectively adsorbed on columns of a specially prepared cation exchange resin. At the same time a large proportion of nitrogenous impurities in the starting material passed through the resin columns into the percolates. The virus could be subsequently eluted from the resin in a fraction of the original volume of

* This work was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported, in part, by the Office of The Surgeon General, Department of the Army, Washington, D.C., and by a research grant from the Lederle Laboratories Division, American Cyanamid Co.

starting material.

Methods. An Amberlite cation exchange resin of the carboxylic acid type, XE-64,† was used. The resin was passed through graded screens and particles between 60 and 100 mesh were placed in a beaker fitted with a mechanical stirrer and converted from the acid to the sodium form with 1-normal sodium hydroxide. The resin was then thoroughly washed with several changes of distilled water. Pyrex glass columns (19 mm I.D.) fitted at the lower end with 1-hole rubber stoppers were charged with 20 ml of the wet resin which was supported on layers of coarse sand and glass wool. The columns were connected to vacuum flasks with rubber tubing. The charged columns were rinsed with distilled water and the resin was then converted to a mixed salt-acid form by treatment with 3 volumes of 1-normal acetate buffer at pH 5.6, the last volume of buffer remaining in contact with the resin for 15 minutes. The columns were again rinsed with 3 volumes of distilled water and drained. Chorioallantoic fluid was collected from 12-day-old chick embryos infected with the PR8 strain of influenza virus, pooled, centrifuged 5 min. at 2000 rpm and stored at —70°C until used. Normal chorioallantoic fluid from chick embryos of the same age was similarly collected and stored. Fifty to 100 ml of fluid was passed through single columns of resin with the aid of negative pressure in 20 to 40 minutes at room temperature and the percolates were collected. The columns were then eluted with four 5 ml portions of 10% sodium chloride and the eluates were collected separately. The percolates and eluates together with the original infected fluids, referred to as control fluids, were saved for various tests. Hemagglutinin titers were determined by a modification of the Salk technic. One-half ml of infected chorioallantoic fluid was serially diluted with saline in 2-fold steps and 0.5 ml of a 1% saline suspension of washed chicken erythrocytes was added to each tube. The endpoint was taken as the last tube in which a pattern was observed after standing at room temperature for 30 minutes. The infectivity of preparations was determined by the chorioallantoic inoculation of 0.2 ml of decimal dilutions of virus into 11- or 12-day-old chick embryos, using groups of 5 embryos for each dilution. Samples of chorioallantoic fluid collected 48 hours later from each chick embryo were tested for the presence of viral hemagglutinin and the results were expressed as the log ID_{50} of the inoculum. Analyses for total nitrogen were performed by the micro-Kjeldahl method. Values for non-protein

† Supplied through the courtesy of Rohm & Haas Co., Philadelphia, Pa.

TABLE I. pH, Total Nitrogen Content and Virus Content of Uninfected and Infected Chorioallantoic Fluids and Resin Percolates and Eluates.

	Vol., ml	pH	Total N, mg/ml	Hemagglutinin titer (recip. dilution)	Infective titer (log dilution)
A.	Uninfected chorioallantoic fluid				
C*	70	8	.55	0	
P	70	9.6	.38	0	
E 1	5	9.5	.43	0	
2	5	9.5	.40	0	
3	5	8.9	.20	0	
4	5	8.4	.13	0	
C	80	8	.63	0	
P	80	10	.39	0	
E 1	5	9.8	.55	0	
2	5	9.6	.45	0	
3	5	8.9	.21	0	
4	5	8.6	.12	0	
B.	Infected chorioallantoic fluid				
C	65	7.6	.54	1024	
P	65	9.5	.40	128	
E 1	5	9.2	.47	1024	
2	5	9.1	.35	8192	
3	5	8.4	.17	1024	
4	5	8	.11	512	
C	60	8.2	.68	2048	
P	60	9.2	.56	256	
E 1	5	9.1	.68	8192	
2	5	8.7	.34	16384	
3	5	8.2	.20	2048	
4	5	8.1	.12	512	
C	100	7.4	.94	2048	6.7
P	100	10	.73	256	4.7
E 1	5	9.5	.92	4096	7.3
2	5	9.5	.86	32768	8.7
3	5	8.9	.49	8192	8
4	5	8.4	.30	512	6.8
C	100	6.6	.52	2048	8.1
P	100	9.1	.39	64	6.8
E 1	5	9	.46	1024	7.7
2	5	8.8	.35	32768	9.2
3	5	8	.21	2048	8.4
4	5	7.7	.19	512	7

* C = control; P = percolate; E = eluate.

TABLE II. Hemagglutinin Titer and Total, Non-Protein and Protein Nitrogen Content of Infected Chorioallantoic Fluids and Resin Percolates and Eluates.

	Vol., ml	Hemagglutinin titer (recip. dilution)	N, mg/ml		
			Total	Non-protein	Protein
C*	50	2048	.74	.38	.36
P	50	64	.47	.25	.22
E 1	5	4096	.56	.27	.29
2	5	16384	.48	.22	.26
3	5	512	.34	.11	.23
4	5	128	.08	.05	.03
C	90	2048	.81	.50	.31
P	90	512	.64	.40	.24
E 1	5	8192	.78	.43	.35
2	5	16384	.54	.25	.29
3	5	512	.31	.14	.17
4	5	128	.22	.12	.10

* C = control; P = percolate; E = eluate.

nitrogen were similarly obtained after sodium tungstate precipitation of proteins. Determinations of pH were made by means of the standard glass electrode method.

Results. Table I(A) shows the results of control tests with normal chorioallantoic fluids, indicating that approximately 60 to 70% of the total nitrogen of these fluids appeared in the percolates and that false positive hemagglutinin reactions were not obtained with the eluate fractions. Table I(B) shows that virus could be concentrated at least 8- to 16-fold in the second eluate fractions, as judged by both hemagglutinin and infectivity tests, even though the total nitrogen content was considerably reduced. Table II indicates that the concentration of virus was accompanied by a relative increase of protein over non-protein nitrogen, although the absolute values were less than in the original fluids.

Discussion. In preliminary tests it was found that concentrated solutions of sodium chloride were necessary to elute the virus from the resin. Sodium and chloride determinations, performed in an attempt to ascertain the possible mechanism of virus elution, showed that very little sodium or chloride appeared in the eluates until nearly all of the virus had been recovered. In the fourth eluates they were present in approximately their initial concentration in the eluting solution. In all of the eluates sodium and chloride were present in approximately equivalent amounts. This would appear to indicate that simple ion exchange was not the sole factor involved in the displacement of the virus from the resin.

From the practical standpoint ion exchange resins may prove helpful in the isolation or concentration of viruses. For example, during the outbreak of influenza in New York City in February-March of 1951, the method was successfully employed to concentrate freshly isolated strains of influenza virus from low-titered chorioallantoic fluids in order to permit their use in hemagglutinin-inhibition tests(3).

Summary. A simple method has been described for the rapid concentration and partial purification of the PR8 strain of influenza virus by means of an ion exchange resin.

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Received March 25, 1952. P.S.E.B.M., 1952, v80.

Development of Fluorine Toxicosis in the Rabbit.* (19511)

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Chronic fluorine toxicosis has been studied in several species including the rat, guinea pig, chicken, rabbit, dairy cow, and human(1-7). It has been shown that small amounts of fluorides cause a retarded growth rate, loss of appetite, and accumulation of fluoride in the bones. Phillips, Hart, and Bohstedt(8) reported that the daily administration to cattle of 2 to 3 mg of fluorine as rock phosphate fluoride per kg of body weight began to show effects after 3 years. A slight loss in weight, loss of appetite and a decline in milk production was evident.

Experimental chronic fluorine poisoning has been reported in the rabbit. Pachaly(9) reported that changes in the maxillary bones occurred in 2 adult rabbits fed several levels of sodium fluoride for extended periods. Largent, Machle, and Ferneau(10) made a study of fluorine poisoning in several mature rabbits fed diets for a period of 16 to 92 days which contained fluorine and they ingested 12 to 50 mg per kg of body weight per day. Microscopic bone changes developed and were presented in detail. The fluoride content of the skeleton greatly increased but no changes were observed on the external structure of the teeth.

It was the purpose of the present study to determine the effect of fluorosis on the growth rate of the young rabbit, a roughage consuming animal, and to determine if there was a similarity of response in this experimental animal to that of the dairy cow fed rock phosphate fluorine.

Experimental. Young rabbits of both sexes weighing approximately 750 g each were distributed and equilibrated to make 8 lots of 5 animals each. They were kept in wire cages on wood shavings and given food and water *ad libitum*. A weigh-back of the left-over food was made daily in order to obtain records of food consumption. The experiment was extended over a period of 4 months. When the experiment was terminated fluorine analyses were made on the tibia and femur from each rabbit by the method of Willard and Winter (11). The control or basal ration consisted of alfalfa 35%, ground oats 25%, soybean oil meal 9.5%, ground yellow corn 24%, wheat middlings 3.5%, salt .5%, and steamed bone meal 2.5%. The fluorides were administered in the form of the rock phosphate borne fluoride or the more soluble sodium salt. The following diets were used:

Lot No.		% fluorine in diet
1	Basal ration or control	.0008
2	" + .625% raw rock phosphate	.022
3	" + 1.25% " " "	.044
4	" + 2.5% " " "	.088
5	" + NaF	.011
6	" + "	.021
7	" + "	.031
8	" + "	.041

These levels of rock phosphate and NaF feeding were based upon previous data obtained with monogastric animals(12). Since NaF fluorine was found to be approximately twice as toxic as the fluorine in rock phosphate it was anticipated that the effects on the animals fed these rations would be roughly paired as follows: Lots 2 and 5, 3 and 6, and 4 and 8. The raw rock phosphate was substituted for a like amount of the steamed bone meal in the basal ration. Sodium fluoride was fed as a direct addition to the basal ration. The bone meal contained by analysis .033% fluorine, while the rock phosphate contained 3.5% fluorine.

Results. The growth rate data for these rabbits are graphically portrayed in Fig. 1. It is clearly evident that the soluble fluoride present as sodium fluoride in the ration of Lots 5 and 6 had very little, if any, effect upon the growth rate of these young rabbits. The fluorine level fed to Lot 2, as rock phosphate fluorine, and Lot 6 as sodium fluoride fluorine are similar in their fluorine content, yet the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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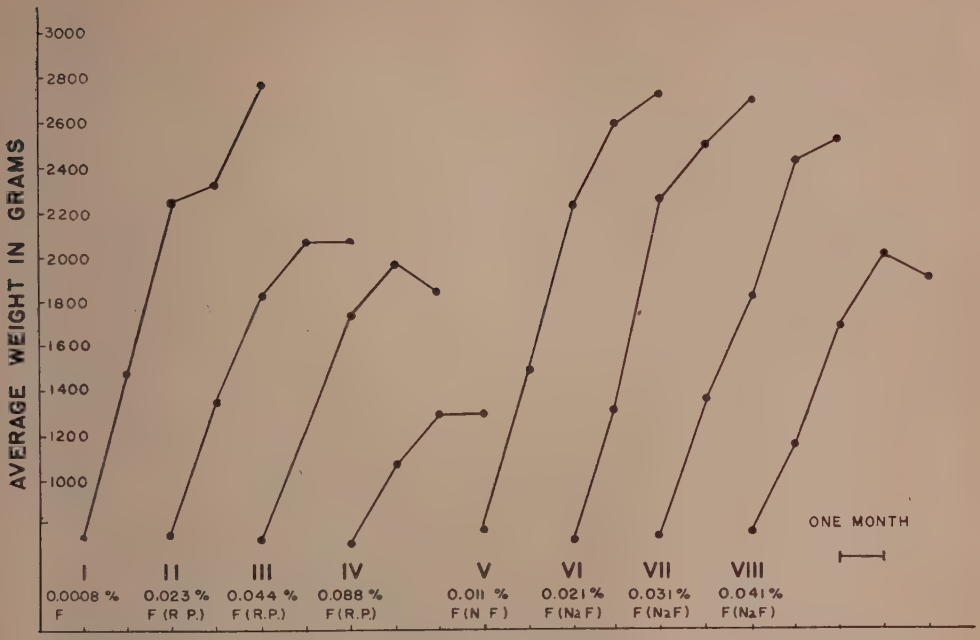

FIG. 1. Effect of various levels of fluorine fed as rock phosphate and as sodium fluoride on the growth of young rabbits.

fluorine in the rock phosphate sharply inhibited growth, while that of the sodium fluoride did not. It was only when the dietary concentration reached .041% fluorine in the form of sodium fluoride that a comparable retardation in growth was obtained equivalent to that of .044% fluorine added as rock phosphate.

Inspection of the data in Table I in part explains the results described above and supports Sollman's appraisal "that fluorine is a protoplasmic poison." The ingestion of fluorine per unit of body weight indicates that the rabbits in Lots 5 and 6 ingested less than 12 mg of fluorine per kg of body weight while those in Lot 2 ingested 13.9 mg of fluorine per kg of body weight. When the ingestion level rose to 16 mg per kg of NaF fluorine there was a lowered average daily gain. The degree of growth retardation in Lots 2 and 7 were alike. These data suggest that .031% fluorine from NaF and .022% fluorine from rock phosphate fluorine are about equal in their effect upon growth. However, .022% fluorine as NaF and .021% fluorine as rock phosphate fluoride caused deposition in the skeleton of nearly equal concentrations, 4360 and 4834 ppm, respectively. The concentration of fluorine in the tibia and femur indicates that the more soluble fluorine of NaF was stored as readily as the rock phosphate fluorine but without an adverse effect upon the growth increment. This fact also may mean that the more soluble sodium fluoride fluorine was ab-

TABLE I. Effect of NaF or Rock Phosphate Fluorine on Growth Increment, Tolerance Levels and Skeletal Fluoride in the Rabbit.

Lot No. and F %		Feed required /100 g gain in body wt, g	Avg daily F ingested, mg	Avg F ingested /day, mg/kg body wt	F content, tibia and femur (dry-fat free), ppm	Avg daily gain in body wt, g
1	Basal	613	.9	.4	275	18.3
2	RP .022	680	22.5	13.9	4360	14.9
3	" .044	765	37.4	23.6	7510	11.1
4	" .088	1090	55.5	50	8938	5.8
5	NaF .011	585	11.7	5.8	3183	18.6
6	" .021	588	21.4	11.3	4834	17.3
7	" .031	680	30.7	16	7075	14.5
8	" .041	735	33.2	22.6	7770	11

sorbed more quickly and deposited in bones much more readily than that of the rock phosphate and hence by this physiologic device did not build up sufficient body concentrations to interfere with growth. At the higher levels the storage was roughly equivalent and growth retardation likewise was equal. Thus, at the low levels of ingestion the skeletal storage was more readily affected by the NaF fluorine and growth was not retarded while rock phosphate fluorine was not stored as readily at the low levels but did retard growth. At the high levels of ingestion the results were much alike in both storage and retardation of growth.

It is apparent from these results that this roughage consuming species was not affected to the same degree as the monogastric animal when the soluble sodium salt was fed as the source of fluorine. This is quite contrary to expectations, and it indicates that the soluble fluoride was not as toxic for the rabbit as it was for the rat when compared to the toxicity of rock phosphate borne fluoride. Rabbits fed the higher levels of fluorine became progressively worse after the first 60 days. This was especially true in Groups 2, 3, 4, and 8, where growth ceased after the third month. The cessation of the growth rate coincided with a very sharply reduced appetite and food intake. At this time these animals were observed to walk with difficulty and exhibited a stiffness in the legs, typical of fluorine toxicosis. The animals receiving .088% fluorine appeared to be in such difficulty as to be practically paralyzed in the hind legs and at the same time became very weak and extremely emaciated.

These data also indicate that since growth in Lots 5 and 6 was not appreciably retarded that the rabbit can withstand 11.3 mg fluorine per kg of body weight per day for the growing rabbit without seriously interfering with the growth process. Apparently increased ingestion above this figure, whether as rock phosphate fluorine or sodium fluoride fluorine, retarded growth in proportion to the increments of fluorine fed. The addition of .022% fluorine (Lot 2) as rock phosphate fluorine resulted in an intake of 22.5 mg per rabbit per day and this amount of fluorine did reduce the rate of growth. This figure then can be taken as being the upper limit of tolerance for the rabbit to rock phosphate fluorine. Further, it is seen that these tolerance levels agree well with the bone storage data obtained with cattle by Phillips, Hart, and Bohstedt(8), in that the bone deposition of fluoride could accumulate without harmful physiologic effect to 5,000 ppm. When the bone fluoride rose to concentrations above 5,000 ppm of fluorine then true fluorine toxicosis developed. On the basis of growth alone it is evident that the minimum toxic level of rock phosphate borne fluorides was below that given to the rabbits in Lot 2. There was less relative difference in the storage between the fluorine of rock phosphate and the sodium fluoride salt, although an increase in the concentration of fluorine in the bones was evident in all lots. Close inspection of these data indicates that proportionately greater storage occurred at the lower levels of fluorine ingestion.

The symptomatology of fluorine toxicosis in the rabbit follows a similar pattern to that observed in cattle. The first evidence of fluorine ingestion was manifest on the surface of the incisor teeth. They lost their pearly, translucent appearance and became white or chalky. This did not affect growth or reproduction at the low levels of fluorine ingestion, but it did at the high levels which were also accompanied by wearing of the molar teeth. This was particularly noticeable in Lots 4, 6, 7, and 8. Gradually, if the levels of fluorine were sufficiently high, stiffness developed due to the exostosis of the leg bones and ankylosis of articulating cartilages. Coincident with this change the bones became a porous, easily penetrable mass in comparison to the compact, tightly formed normal bone.

Summary and conclusions. A study of growing rabbits fed a complete diet supplemented with various levels and forms of fluorine has been made. When levels of fluorine were included in the ration above .021% rabbits in 4 months developed a symptomatology indicating the development of fluorine toxicosis. Growth was retarded, stiffness of the joints occurred, and pronounced changes in the teeth and structure of the bone were encountered. Fluorine was concentrated in the skeleton, and it appears from

these data that the rabbit can withstand a storage of fluorine up to about 5,000 ppm without affecting such physiological processes as growth, health, normal teeth, and bone. In this species the fluorine of raw rock phosphate produced as toxic results, or more so, as an equivalent level of sodium fluoride borne fluorine.

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Received March 31, 1952. P.S.E.B.M., 1952, v80.

Anemia of Infection. XVIII. Effects of Turpentine and Colloidal Thorium Dioxide on Rat Plasma Iron Levels.* (19512)

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It was reported previously from this laboratory(1,2) that the intramuscular injection of turpentine into either rats or dogs is followed by transient hypoferremia. It was subsequently demonstrated in dogs that the intravenous injection of a single dose of colloidal thorium dioxide (CTD) is followed by a decrease in the plasma iron, whereas after the administration of three consecutive daily intravenous injections of CTD there is an appreciable increase in the plasma iron above the normal level(3). Following the repeated injection of CTD the hypoferremia-producing effect of turpentine is abolished. Since both iron and CTD are in large part removed from the circulation by the macrophagic tissue, it was suggested that one possible explanation of the above observations is that the injection of turpentine in some way enhances the uptake of iron by these cells and that the repeated administration of CTD temporarily impairs this ability.

The purpose of the present paper is to describe observations dealing with the effect of the intravenous injection of various amounts of CTD and of injections of CTD together with turpentine on the plasma iron of the adult male albino rat.

Methods. Approximately 380 adult male Sprague-Dawley rats weighing 250-300 g were used in these experiments. The diet consisted of Purina dog chow pellets. A 25% suspension of colloidal thorium dioxide (Thorotrast, Heyden Chemical Corporation, New York City, N. Y.) was given intravenously in the amounts indicated in the various experiments. The femoral vein was used for intravenous injections. This vein was surgically exposed under light ether anesthesia and injections were made with a 1 ml tuberculin syringe and a 26 gauge needle. Clean, but not sterile, technic was observed throughout the operative procedures. When hemorrhage occurred as a result of the injections, the animal was discarded from the experiment. The turpentine (0.1 ml/100 g body weight, rectified oil of turpentine, Rexall, USP) was injected into the musculature of the hind leg. The animals were sacrificed by exsanguination

* This investigation was supported by a research grant from the National Institutes of Health of the U. S. Public Health Service.

† Fellow of the Damon Runyon Memorial Fund for Cancer Research.

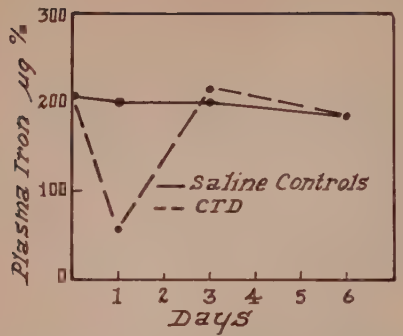

FIG. 1. Effect on plasma iron of a single intravenous inj. of 4 ml of colloidal thorium dioxide (CTD) per kg of body wt, given at zero time. Each point represents the mean of 10 rats.

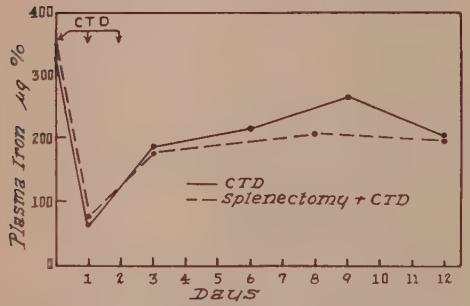

FIG. 2. Effect on plasma iron of intravenous inj. of colloidal thorium dioxide (CTD) (2 ml/kg of body wt) given on days 0, 1 and 2 (solid line). The results are compared with the effect of a similar amount of CTD given to rats splenectomized 3 weeks previously (broken line). Each point on the solid line represents the mean of 10 rats. Each point on the broken line represents the mean of 5 rats.

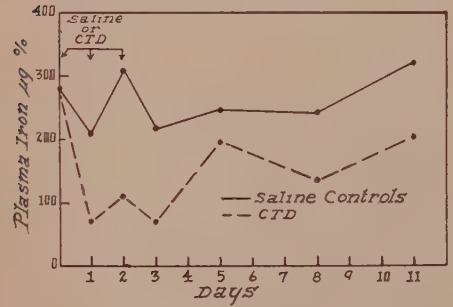

FIG. 3. Effect on plasma iron of the intravenous inj. of 6 ml of colloidal thorium dioxide (CTD) per kg of body wt, given on days 0, 1 and 2. The control animals were given 6 ml of saline per kg of body wt on days 0, 1 and 2. Each point represents the mean of 10 rats.

from the abdominal aorta. The blood was placed in centrifuge tube containing 0.1 ml of 20% potassium oxalate, was immediately centrifuged and the plasma removed. *Plasma iron determinations* were made according to a modification of the method of Barkan and Walker(4) in which the Beckman spectrophotometer was used as described in a previous publication(5).

Experimental. The effect of a single intravenous injection of 4 ml/kg of body weight of CTD on the plasma iron is compared in Fig. 1 with the effect of a similar amount of saline injected intravenously in control animals. It will be noted that the injection of CTD caused a profound hypoferremia at 24 hours. Thereafter the plasma iron level increased promptly to normal.

The effect on the plasma iron of three daily intravenous injections of 2 ml CTD per kg of body weight is presented in Fig. 2. Twenty-four hours after the first injection of CTD there was a marked hypoferremia. However, this degree of hypoferremia did not persist in spite of the further administration of CTD.

With the object of surgically removing a portion of the reticuloendothelial system, 20 rats were splenectomized and 3 weeks later three daily intravenous injections of 2 ml CTD per kg of body weight were given. As shown in Fig. 2, the hypoferremia which resulted was no more pronounced in degree nor more prolonged in time than that which followed the administration of CTD alone.

To determine whether a total dose of CTD greater than 2 ml/kg of body weight would result in hyperferremia rather than hypoferremia, 60 rats were given 3 daily intravenous injections of 6 ml of CTD per kg of body weight. At the same time 60 control rats were given 3 daily intravenous injections of 6 ml of saline per kg of body weight. The results are presented in Fig. 3. During the period of CTD administration and for the first 24 hours thereafter, there was a marked reduction in the plasma iron level. Following this the plasma iron level increased somewhat but continued to remain significantly below the level of the control animals.

In order to learn whether the hypoferremia-producing effect of turpentine would be blocked by the prior administration of CTD,

TABLE I. Effect of Turpentine on Plasma Iron Level of Control Rats and of Rats Treated Previously with Colloidal Thorium Dioxide (CTD).

Pretreatment	Plasma iron,* μg %	Treatment†	Plasma iron‡ μg %	Plasma iron‡ % decrease
Saline, 3 ml/kg × 3	207 ± 11	Turpentine	81 ± 4	61
CTD "	156 ± 6	0	164 ± 6	0
		Turpentine	96 ± 6	39
Saline, 6 ml/kg × 3	232 ± 6	Turpentine	69 ± 6	70
CTD "	142 ± 10	0	109 ± 6	23
		Turpentine	104 ± 7	27

* One hr after last "pretreatment" inj. † Given 1 hr after last "pretreatment" inj.
‡ Twenty-four hr after treatment.
Figures represent the mean ± stand. error of 15 determinations.

two groups of 45 rats each were given 3 daily injections of CTD intravenously. One group received 3 ml/kg and the other group was given 6 ml/kg of body weight daily. Fifteen rats in each group were sacrificed one hour after the last injection in order to obtain a baseline value for the plasma iron. An equal number of rats in each group was given intramuscularly .1 ml turpentine per 100 g of body weight at this time. Twenty-four hours later the animals injected with turpentine as well as 15 rats in each group given only CTD were sacrificed. As controls 2 groups of 30 rats each were given 3 daily injections of saline intravenously. One group received 3 ml and the other 6 ml/kg of body weight. One hour after the last injection 15 rats in each group were sacrificed and the plasma iron was determined. The remaining animals were given .1 ml turpentine per 100 g of body weight and were sacrificed 24 hours later. The results are summarized in Table I.

The administration of turpentine to animals previously given saline resulted in a marked decrease in the plasma iron level at 24 hours. The administration of turpentine to rats which had been pretreated for 3 days with 3 ml CTD per kg of body weight resulted in only a 39% decrease in the plasma iron. Animals pretreated with twice the above dose of CTD failed to develop a further reduction in the plasma iron level as a result of turpentine administration; the plasma iron level was not significantly different from that of the animals receiving only CTD.

Discussion. In confirmation of work in the dog reported previously from this laboratory, it has been observed in the rat that following the repeated administration of colloidal thorium dioxide, the hypoferremia-producing effect of turpentine is appreciably decreased. However, in the dog it was observed that three consecutive daily intravenous injections of 2 ml CTD per kg of body weight was followed by a marked increase in the plasma iron level. In the rat the administration of 3 consecutive daily intravenous injections of 2, 3 or 6 ml CTD per kg of body weight, either to intact or to previously splenectomized animals, resulted in a persistent mild hypoferremia rather than hyperferremia. The duration of the hypoferremia seemed to be related to the amount of CTD administered, the larger amounts causing a more prolonged hypoferremia.

These studies may be compared to those of previous workers, although the conditions of the experiments were not similar since different species of animals were used and the amount and rate of CTD administration differed. Thus, Barkan(6) administered CTD to rabbits in a single injection in amounts ranging from 0.37 to 1.1 ml/kg of body weight and observed hypoferremia 24 hours later. The administration of 8.2 ml CTD per kg of body weight over a 10-day period failed to alter significantly the plasma iron level when determined 7 days after the last CTD injection(7). Thöenes and Aschaffenburg(8) gave single injections of CTD to rabbits and found hypoferremia at 24 hours with a return towards normal of the plasma iron level 48 hours after the injection. Vannotti and Imholtz(9) observed a decrease in the total "non-hemoglobin" iron in rabbits following single injections of CTD and after repeated injections at short intervals. The administra-

tion of CTD to splenectomized animals, contrary to our observations in the rat, resulted in a marked increase in the "non-hemoglobin" plasma iron fraction.

Summary. Single intravenous injections of colloidal thorium dioxide in the rat produced a marked transient hypoferremia. A more persistent hypoferremia resulted from the administration of this substance daily for 3 days both in intact and in splenectomized rats. The hypoferremia producing effect of turpentine was markedly decreased by the prior administration of colloidal thorium dioxide.

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Received April 1, 1952. P.S.E.B.M., 1952, v80.

Effect of Total Body X-Irradiation on Plasmin Inhibitor Titer in Blood of Rats. (19513)

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It has been previously reported that a decrease in the plasmin inhibitor titer could be demonstrated in the plasma of rats subjected to "tourniquet shock" or after epinephrine administration(1). Since it has been established that acute ionizing radiation illness is associated with pronounced hemorrhage, it was of interest to study the effect of total body x-irradiation on the plasmin inhibitor titer in blood. The results obtained are recorded in this communication.

Experimental. Male rats of the Sprague-Dawley strain, weighing 210-325 g were kept in individual cages and were weighed daily prior to and during the experiment. Three groups of animals were used: Group I (44 animals) served as normal controls for Groups II and III. The animals were maintained on Purina laboratory chow and tap water until sacrifice. Group II (166 animals) consisted of the irradiated animals. The rats were irradiated, 2 at a time, in a well-ventilated lucite chamber. The radiation was performed with a 250 Kv Kelly-Koett x-ray unit, the factors being: 250 Kv, 6 ma, ½ mm copper and 1 mm aluminum filters, target distance 29 cm. The dosage was 40 roentgens per minute.* Each rat received either 880 r (22 min.) or 1000 r (25 min.); 880 r was found to correspond to an LD/78 (28 days), and 1000 r to an LD/100 (8 days). Since it is known that the food intake of irradiated animals is less than normal, it was desirable to study the effect of starvation on the plasmin inhibitor titer. In Group III (69 animals) food was withheld, but the animals were allowed tap water. On days 0, ¼, 1, 2, 3, 4, 5, and 6, 2 ml of blood was drawn from the animals by direct cardiac puncture using 0.2 ml 3.8% sodium citrate as an anticoagulant. Following centrifugation at 2800 rpm for 45 minutes, the plasma was withdrawn and tested immediately or stored sealed in the refrigerator for use later the same day. Determinations of antifibrinolytic activity were carried out similarly as described by Guest, Ware, and Seegers(2). 0.1 ml of plasma was diluted 1:40 with pH

* The authors express their appreciation to the Radiobiology Branch of this laboratory for assistance in the irradiation procedures.

7.25 imidazole† buffer. 0.1 ml of this solution was allowed to react 30 minutes at 25°C with 0.1 ml of previously standardized trypsin.‡¶ The tube was then transferred to a 37.5°C bath and 0.1 ml thrombin§ (10 units) and 0.2 ml bovine fibrinogen‖ were added. The end point as determined by bubble rise in the lysed clot was read in seconds. These were converted into units of plasmin inhibitor from a standard curve(1).

Results. The results of the above described experiments are discussed on the basis of the effect of either total body x-irradiation or starvation on the antiplasmin titer, on weight, and on mortality. Each point used in the construction of Fig. 1 represents the average

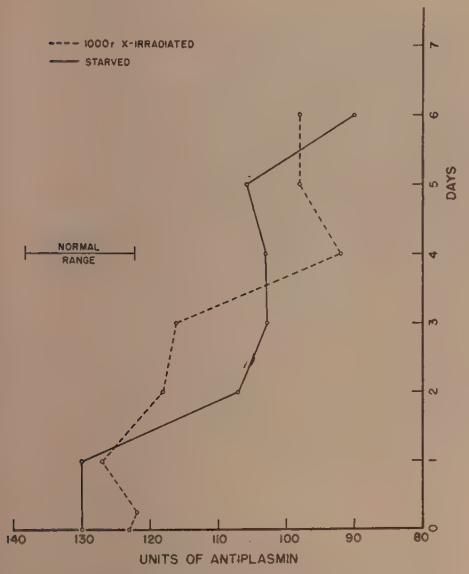

FIG. 1. Change in antiplasmin titer following x-irradiation and starvation.

† Obtained from Edcan Laboratories, Norwalk, Conn.

‡ Worthington Biochemical Laboratories, Freehold, N. J.

¶ Standardized Trypsin: The amount of trypsin which will dissolve a 0.1% fibrin clot in a total volume of 0.5 ml within 120 seconds at 37.5°C at pH 7.25.

§ Parke-Davis Co., Detroit, Mich.

‖ Fibrinogen prepared according to Reference(3). Concentration in the 0.5 ml standard clot = 500 γ or 0.1%.

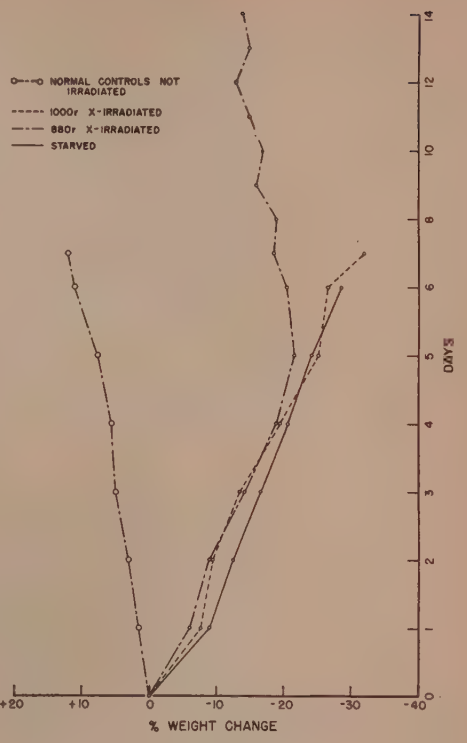

FIG. 2. Change in weight following x-irradiation and starvation.

of separately assayed plasmas of at least 10 rats. As can be seen from Fig. 1, the antiplasmin titer of the irradiated (1000 r) rats** started to decrease 2 days after irradiation, reached a minimum in 4 days, and then increased slightly on the 5th and 6th days after irradiation. Starvation produced a somewhat similar lowering, reaching a minimal value in 6 days as compared to a minimum in 4 days in the irradiated rats. Apparently the fall in the plasmin inhibitor titer is more abrupt in the irradiated than in the starved animals. In this connection reference may be made to the findings presented by Cronkite(4) suggesting that serum plasmin formation may occur in acute radiation illness in goats and swine.

** Rats irradiated with 880 r showed after 14 days an antiplasmin titer of 106 units (average of 9 survivors out of 20 irradiated animals), comparable to the lowering obtained after irradiation with 1000 r in 3½ days.

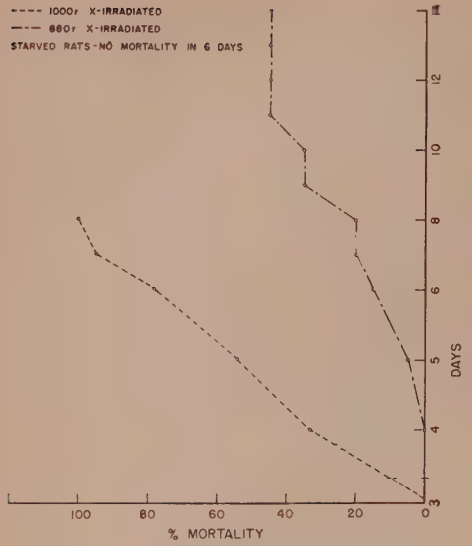

FIG. 3. Mortality following x-irradiation.

As can be seen from Fig. 2, irradiated (880 r and 1000 r) and starved animals lost weight at the same rate through the 7th day. In the group receiving 880 r, the survivors (about 50%) slowly regained weight from the 5th day. These observations are in agreement with those reported by Smith, Tyree, Patt, and Jackson(5).

It is interesting to note (see Fig. 2 and 3) that although the mortality differs greatly among the 3 groups, the weight loss up to 5 days is very nearly identical.

Conclusions. Exposure of rats to 1000 r total body x-irradiation was followed by a fall in the plasmin inhibitor titer of plasma, beginning at the second day after irradiation and then gradually increasing. Since starvation was found to exert a similar effect, no definite conclusions can be made as to whether irradiation *per se* caused a lowering in the titer. Total body x-irradiation (1000 r) and starvation caused a weight loss of nearly identical magnitude.

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Received April 2, 1952. P.S.E.B.M., 1952, v80.

Crystalline Components of Catalase. (19514)

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The activities of crystalline catalase preparations have been found to vary considerably. Employing amino acid analysis and immunological tests Bonnichen(1) found that the protein component of horse erythrocyte and liver catalase are identical. The investigations of Theorell(2), using radioactive iron, indicate that catalase of the liver and of the blood are of different origins, but that their albumin components are the same. It has recently been found by solubility technics that beef liver catalase contains one fraction having a Kat. F. as high as 180,000(3). It is generally assumed that there exists, in nature, a number of catalase molecules with different activities(3). Beef liver catalase has been shown to crystallize in the form of long arrow shaped plates(4), and several other forms(5), and beef erythrocyte catalase may be obtained in the shape of characteristic prisms having hexagonal bases(4,6).

In the present report we wish to describe the preparation of cow liver catalase consisting of a mixture of long, thin plates, and prisms with hexagonal bases; the latter are similar to those obtained by Salkowski and Sumner

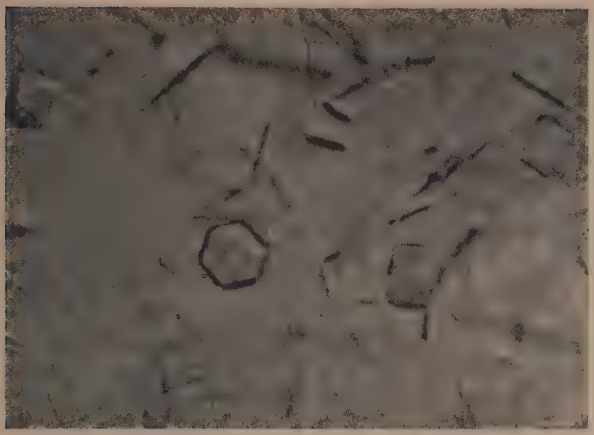

FIG. 1. Crystaline mixture of cow liver catalase. 320 ×.

FIG. 2. Prisms with hexagonal bases; component of cow liver catalase. 3200 ×.

from beef erythrocytes(4,6). Our observations appear to support the view that there exist, together in the same tissue, different catalase molecules.

Experimental. Crystalline cow liver catalase was prepared according to the procedure of Tauber and Petit(5). The catalase was recrystallized by dissolving it in the least volume of 0.01 N sodium hydroxide. Without centrifuging, the solution was immediately adjusted to pH 5.8 by the addition of the calculated volume of 0.01 N acetic acid. A small quantity of insoluble matter was removed by centrifuging at room temperature. The clear supernatant was placed in a refrigerator at 4°. In about 3 hours the catalase began to crystallize rapidly in the form of microscopic, long, thin plates and some prisms having hexagonal bases (Fig. 1 and 2). The prisms had exactly the same appearance as the crystals obtained by Salkowski and Sumner from beef erythrocytes(4,6). The activity of our crystalline mixture, as found by the method of Euler and Josephson (7), was 34,000 Kat. F.

This method is applicable to the recrystallization of commercial (Worthington) catalase, which apparently does not retain its crystalline state in the ammonium sulphate solution. From such an amorphous suspension of beef liver catalase, which was kept for 3 years at 4°, the catalase was collected by centrifug-

ing or by removal of the salt by dialysis prior to centrifuging. Recrystallization was readily affected by the procedure just described.

Summary. A simple method for recrystallizing catalase has been described. Some of the cow liver catalase crystals present in the mixture have exactly the same shape as those of beef erythrocyte catalase. This fact points to a multi-molecular nature of cow liver catalase. Since it is difficult, however, to remove all of the blood present in liver, it is quite possible that the described prisms originate from erythrocytes and that the higher active component described by other investigators may have a similar origin.

The author is grateful to Dr. W. E. Vannier for preparing the photomicrographs.

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Received April 2, 1952. P.S.E.B.M., 1952, v80.

Initiation of the Clotting of Blood.* (19515)

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It was recently observed that when hemophilic blood was put in a glass tube which previously had contained a solution of thrombin, but which had been rinsed repeatedly with saline solution, clotting was considerably faster than in a control test tube that had contained only saline(1). Since this simple experiment serves as a useful means for obtaining salient information concerning various factors that play a role in the clotting of blood, it was repeated with certain modifications as recorded in Table I.

From the recorded results, it can be seen that hemophilic blood clotted almost completely in 10 minutes when placed in a test tube which had contained thrombin but which had been repeatedly rinsed with saline. The same blood in a control test tube rinsed only with saline clotted in 40 minutes. Platelet-rich plasma obtained from the same hemophilic subject likewise formed a semi-solid clot in 10 minutes in a test tube that had contained thrombin, whereas the same plasma depleted of platelets by high centrifugation required 90 minutes before a clot of similar density was formed.

Obviously the primary cause of the marked shortening of the clotting time in the test tube that had contained thrombin was a minute amount that remained. Since the tube had been rinsed repeatedly, it seems likely that the thrombin was adsorbed to the glass surface probably as a thin film. Since the platelet-poor plasma clotted very slowly, although the tube contained the same amount of residual thrombin, it is clear that the minute quantity of adsorbed thrombin was not enough by itself to account for the accelerated clotting. Since the platelet-rich plasma, like the whole blood, clotted rapidly, one may conclude that more thrombin was formed in this plasma, which accounts for the decreased clotting time. Since this did not occur in the platelet-poor plasma, it seems evident that the platelets participated in increasing the forma-

* This work was supported by a grant from the Division of Research Grants, National Institute of Health, USPHS.

TABLE I. Adsorption of Thrombin on Glass and Its Influence on the Clotting Time of Hemophilic Blood and Plasma.

	Surface of test tube exposed to			
	Saline only	Thrombin*		
	Blood†	Blood†	Plasma†	
			Platelet-rich	Platelet-poor
Semi-solid clot	30 min	10 min	10 min	90 min
Solid clot	40	20	20	120
Prothrombin consumption test‡	8 sec	8 sec	8 sec	8 sec

* One cc of a concentrated solution of thrombin was transferred into small test tubes (13 × 100 mm). After 1 min the solution was poured out, the test tube drained, then filled with saline solution, and again drained. The rinsing and draining was repeated 4 times.

† Blood was collected with a silicone-coated syringe and needle. The platelet-rich plasma was prepared by centrifuging blood at 800 r.p.m. 5 min, while the platelet-poor plasma was obtained by centrifuging the blood 10 min at 8000 r.p.m. The latter plasma contained approximately 3000 platelets per mm^3. The centrifuge tubes were coated with silicone, and the blood chilled in an ice bath. For determination of the clotting time, 1 cc of blood or plasma was transferred to the test tube which was placed in a water bath at 37°C.

‡ The reagents and tests employed were carried out as described recently(1). These studies were repeated on the bloods of several hemophiliacs.

tion of thrombin. It may be postulated that an interaction of thrombin and platelets caused the generation of more thrombin. Actually, the additional amount of thrombin formed, which was sufficient to bring the clotting time nearly to normal, must have been extremely small since no utilization of prothrombin was demonstrable with the prothrombin consumption test.

These findings clearly show that an extremely small quantity of thrombin is sufficient to inaugurate *in vitro* clotting even of hemophilic blood. The results further indicate that thrombin in some manner interacts with platelets to bring about the formation of additional thrombin. One may assume that this is presumably an autocatalytic reaction which in hemophilia is limited by the lack of a plasma factor which has been designated as thromboplastinogen. Because of the marked effect of an extremely minute amount of thrombin on the clotting time, it is easy to understand why a faulty veni-puncture may introduce enough tissue juice which is rich in thromboplastin to reduce the clotting time to such an extent that a false normal value may be obtained. Furthermore, in the clotting of hemophilic blood a solid gel may form before less than one-third of the fibrinogen has been changed to fibrin. Thus, the clotting time is not necessarily a true measure of complete coagulation, and a normal value is no assurance that the clotting reaction is quantitatively normal. To have any significance, the clotting time must be meticulously standardized since seemingly small deviations in technic such as using improperly cleaned test tubes may induce serious errors. It is to be emphasized that in studying the clotting time in test tubes, one introduces artificial factors such as foreign surfaces and interphases that are absent in physiological *in vivo* clotting.

Summary. A minute amount of thrombin such as remains adherent to the wall of a test tube after repeated rinsing is enough to shorten markedly the clotting time of blood from a severe hemophiliac. Since the same effect is observed in platelet-rich but not in platelet-poor hemophilic plasma, it appears that although an extremely small quantity of thrombin can initiate clotting, platelets participate in accelerating the reaction.

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Received April 3. 1952. P.S.E.B.M., 1952, v80.

Zone Electrophoresis in a Starch Supporting Medium. (19516)

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Recent investigations employing filter paper have demonstrated the value of electrophoresis in a supporting medium(1,2). The complete separation of components, rather than the partial separation occurring in the unpacked channel of the Tiselius cell, makes this method useful for preparative purposes. However, certain limitations of the filter paper technic have become apparent. First, the capacity is low making it difficult to isolate substantial amounts of material. Second, adsorption and consequent trailing has been noted with certain substances, particularly the basic proteins and peptides. Third, elution of material from the paper may be difficult and low recoveries are often obtained. As a result, a search was made for other types of supporting media that might be free of these disadvantages. Certain of these had previously been used for similar purposes(3,4).

Among the materials investigated were glass beads, glass powders, special sands, gels, acid and basic resins and starch. Some separation of applied protein could be achieved when each of these materials, mixed with buffer, was molded into a block and placed in an electric field. The block was made just dry enough to preserve its form and was encased in wax paper. A very large electroosmotic flow of water to the cathode was noted in most cases and represented a disadvantage. Albumin, colored yellow by excess bilirubin bound to it, and hemoglobin, were used to test the separation and the electroosmotic flow. No separation of these 2 proteins was obtained by simple water flow through the medium without an electric current. The electroosmotic flow could be calculated for each supporting medium from the relative position of each of the two colored proteins to the origin and to each other(2).

The approximate relative mobility of electroosmotic flow could also be calculated from the observed mobilities of these 2 proteins in free solution, 6.5 in the case of albumin and 3.4 in the case of hemoglobin (Table I). In some instances the flow of liquid to the cathode was almost as great as the migration of albumin to the anode, resulting in an almost stationary position for this protein. Adsorption was tested with a solution of lysozyme and β_1 lipoprotein which had been found to adsorb to filter paper leaving a trail. In general, considerable adsorption of these substances was observed with those materials giving a high electroosmotic flow. This could be observed by simply shifting the protein by passing buffer through the block with a siphon-like arrangement without an electric current, or from the trailing in an electric field. Fig. 1 shows the absence of adsorption of lysozyme on starch as compared with Whatman 3 MM filter paper. On filter paper this preparation of crystalline lysozyme gave such trailing that it was impossible to determine the electrophoretic homogeneity. No significant adsorption was observed with the starch for any of a large group of proteins and peptides that were tested. Because of this finding, and in view of the relatively low electroosmotic flow, it was employed as the main supporting medium for subsequent studies.

TABLE I. Electroosmotic Flow for Various Media Expressed in Terms of Mobility (μ_{e1}) and in Relation to the Observed Distance of Migration of Albumin. (Barbital buffer pH 8.6, μ .1).

Medium	$\mu_{e1} \times 10^5$	$\frac{d_{e1}}{d_{alb}}$
Filter paper (Whatman 3 mm)	1.5	.30
Potato starch (Amend)	2.5	.62
Washed sea sand	4.3	1.95
Ground glass (Corning 35-60 mesh)	5.1	3.64
" " (" 150-200 ")	5.9	9.87
Soft glass beads (200 mesh)	5.6	6.20
Agar (1%)	4.7	2.60

Fig. 2 illustrates a barely moist block of starch* encased in wax paper between 2 glass plates resting on the lips of electrode vessels. Contact of the starch block with the buffer solution was obtained with plastic sponges or moist cloth. After the starch block had been

* Amend potato starch.

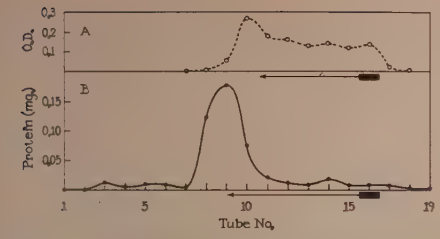

FIG. 1. Curves obtained for lysozyme employing electrophoresis with filter paper (A) and starch (B) in phosphate buffer at pH 7.6, μ.1. Protein in A was determined by the bromphenol blue elution method on paper segments, in B by the modified Folin procedure.

FIG. 2. Photograph of electrophoresis apparatus with a starch block between glass plates resting on the lips of electrode vessels. Contact with the buffer vessels was made through plastic sponges.

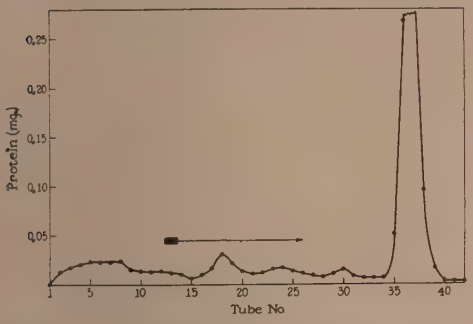

FIG. 3. Curve obtained for a normal serum separated in the starch medium with analyses for protein on the saline extract of the starch segments by the modified Folin method. The arrow and block indicate the origin and direction of migration. Barbital buffer pH 8.6, μ.1.

formed by pouring the starch solution into a mold lined with wax paper, permitted to settle and partially dried with heavy filter paper, the protein was applied either directly with a pipette into a narrow slit in the starch block or by removing a rectangular segment of starch and replacing it with a barely moist mixture of protein and starch. Although various sizes of the starch block were employed, a 38 x 10 x 1.5 cm size was used most frequently. Following the conclusion of an experiment (usually 24 hours at 400 volts at low temperatures) the block was dried slightly, cut into 1 cm segments, shaken with saline and aliquots taken after the starch had settled. Good recoveries could be obtained by displacement filtration of the starch on a sintered glass funnel under suction.

In most experiments employing the starch block, 1-5 cc of material were applied to the origin. Fig. 3 illustrates the pattern obtained for 1 cc of normal serum separated in a 50 cm block. An aliquot representing 4% of the material from each starch segment was used for protein analysis by the modified Folin tyrosine method(2). This was found to have some advantage over the ninhydrin method although both were used. The main components observed by classical free electrophoresis can be seen. The heterogeneity of the gamma globulin is quite apparent. Since only 4% of the material of each tube was used for the protein curve, the remainder was available for other analyses. The best patterns for serum were obtained with volumes below 2.5 cc. Larger volumes presented more difficulties but some separation could be obtained. The α_1 peak often was not visible when more than 4 cc of serum were used. Curvatures in the bands represented a limiting factor of the technic and became more pronounced with larger volumes of serum. This could be seen from the natural pigments of serum, particularly the yellow albumin, when a light was placed under the starch block. Three pigment bands were usually observed for normal human serum. The best results were obtained with serum diluted to ⅔ concentration or less with buffer.

The major application of the zone electrophoresis method in the present study was to the problem of the lipoproteins of serum. The α and β lipoproteins were readily separated in the starch medium and could be followed by cholesterol and phospholipid analyses on the starch segments. Fig. 4 illustrates the cholesterol and phospholipid curves obtained

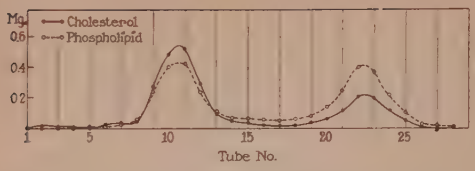

FIG. 4. Curves obtained by cholesterol and phospholipid analyses of starch segments for a normal serum. The two peaks represent the α (tube 22) and β (tube 11) lipoproteins with different cholesterol-phospholipid ratios. Barbital buffer pH 8.6, μ .1. Origin in tube 8.

for an essentially normal serum at pH 8.6, μ 0.1. The high cholesterol-phospholipid ratio for the β-lipoprotein and the lower ratio for the α-lipoprotein are apparent. A further subdivision of these lipoproteins was obtained at lower pH and ionic strength. Trailing of the β-lipoproteins was obtained with various types of filter paper and glass powders, but was not observed with the starch. The details of the lipoprotein studies have been reported separately(5).

Discussion. Certain of the other supporting media that were tested possessed specific advantages over the starch. For example, agar gave a very low supporting medium—liquid ratio, an important factor in increasing the capacity of the system. The starch system usually gave a ratio of approximately 1, with the starch occupying one-half of the total volume. However, difficulties with the agar system, particularly in isolating components at reduced temperatures, overcame this advantage. The filter paper system possessed the advantage of the extreme sensitivity and simplicity of the protein staining technic with bromphenol blue. No success was achieved in adapting this to the starch. A further disadvantage of the starch was that certain impurities sometimes interfered with specific analyses on the segments, although some of these, such as the ninhydrin positive materials, could be washed off. Starch did not represent the ideal supporting medium but because of the homogeneous packing achievable, the lack of adsorption, good recoveries and the relatively low electroosmotic flow, it proved useful for many purposes. Further advantages have been derived with the starch system in narrow vertical columns employing a liquid displacement method rather than the segmentation technic.

Summary. 1. A procedure was described for the electrophoretic separation and isolation of materials employing various types of supporting media. 2. Starch proved particularly useful because of its low adsorption of proteins and peptides in aqueous buffers. 3. A comparison was made of the electroosmotic flow in various media under similar conditions. 4. The separation of 1-4 cc of serum with isolation of the components could be carried out employing the starch system. 5. The α- and β-lipoproteins of serum could be determined quantitatively by phospholipid and cholesterol analyses of the serum fractions.

The authors are indebted to Dr. Stanford Moore, Dr. William H. Stein, and Dr. Lewis G. Longsworth for helpful suggestions regarding supporting media.

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Received April 7, 1952. P.S.E.B.M., 1952, v80.

Determination of Salicylic Acid and Related Substances in Serum by Ultraviolet Spectrophotometry.* (19517)

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A variety of methods are available to the clinician and the research worker for estimating salicylic acid and its derivatives in biological fluids(1-6). Any new technic, therefore, has to present some definite advantage over the existing ones. The present paper describes a simple and specific method based on ultraviolet absorption of salicylic acid and suggests similar technics for the estimation of related substances.

Methods. Absorption measurements were made on the Beckman Quartz Spectrophotometer, model DU, with ultraviolet attachment, using a hydrogen discharge tube as the source of radiation. The solutions were placed in silica cuvettes with a light path of 10 mm ± 0.05. Measurements were made against blanks containing all the reagents except the substance to be tested. Guinea pig blood was used for salicylate determinations. The animals were injected intraperitoneally with varying doses of sodium salicylate, and cardiac blood samples were taken prior to and one hour after injection. The blood was allowed to clot and the serum was treated as described below.

Salicylate determination. The ultraviolet spectrum of a 10^{-4}M solution of sodium salicylate in water (pH = 5.1) has an absorption peak at 295 mμ. The molar extinction coefficient at this wavelength,† $E_M^{295} = 3840$. However, when sodium salicylate is dissolved in serum and the protein precipitated with trichloracetic acid, the filtrate shows a different peak (λ = 303 mμ) and a slightly different extinction coefficient ($E_M^{303} = 3680$). This difference is caused by the change of pH and not by the presence of serum. Addition of the same amount of trichloracetic acid as in the previous experiment, to sodium salicylate results in a change in pH to 1.2 and also in a shift of the peak to 303 mμ. Fig. 1 shows the two spectra of sodium salicylate, with wavelength plotted against molar extinction coefficient. For determination of salicylate the following procedure was used: The serum (or plasma) is pipetted into a centrifuge tube. Using 0.5 ml is satisfactory in most cases except when high salicylate levels are expected in which case smaller quantities should be used. The volume is made up to 5 ml with a 4% solution of trichloracetic acid. After an interval of at least 30 min the mixture is filtered or centrifuged. The clear supernatant is poured into a silica cuvette and the absorption measured at 303 mμ in the ultraviolet spectrophotometer. The test solution should be read against a blank consisting of the tri-

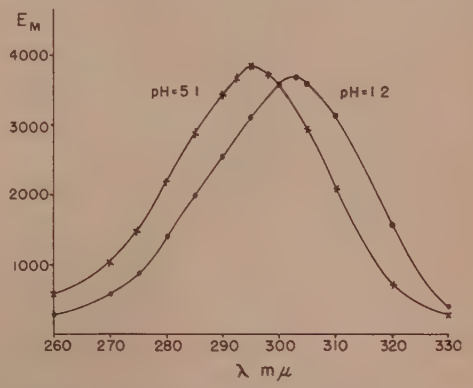

FIG. 1. Ultraviolet absorption spectrum of sodium salicylate dissolved in water (pH = 5.1) and mixed with trichloracetic acid (pH = 1.2). λ = wavelength in mμ; E_M = molar extinction coefficient.

* This work was supported by a grant from the Walter P. Murphy Foundation.

† The molar extinction coefficient, $E_M^{\lambda} = \frac{\log \left\{ \frac{Io}{I} \right\}}{cl}$ for a particular wavelength (λ). c = the concentration expressed as moles/liter; l = length of the light path in the cell in cm; Io = intensity of the incident light and I = intensity of the transmitted light; $\log \left\{ \frac{Io}{I} \right\}$ = (O.D.), optical density.

TABLE I. Comparison Between Blood Salicylate Levels Obtained by the Ultraviolet Method and a Colorimetric Technic (3).

Na salicylate administered, mg/kg	Serum salicylate, mg/100 ml — Ultraviolet method	Colorimetric method
150	19.15	15.9
	22.55	25.9
	24.15	24.7
		25.2
		25.4
	Mean=21.95±1.5	Mean=23.3±1.9
200	26.05	25.7
	28.7	28.6
	29.1	30.8
	29.55	
	29.55	
	30.7	
	Mean=28.9 ± .6	Mean=28.4±1.5
250	36.5	30.5
	41.2	36.5
	38.7	36.9
		42.1
	Mean=38.8 ±1.4	Mean=36.5±2.4

chloracetic filtrate of the serum of the same subject before administration of salicylate. When this is not practicable a blank containing equivalent quantities of saline and trichloracetic acid can be used. In this case, the mean absorption of serum should be subtracted from the optical density reading. The mean absorption can be determined on a series of sera from normal untreated subjects.

The results can be calculated from the following formula: Sodium salicylate mg/100 ml serum $= \frac{(O.D.)}{\frac{E_M}{(M.W. \times 10)}} \times \frac{V}{v}$ where (O.D.) = optical density (against serum blank or after subtraction of serum absorption); (M.W.) = molecular weight of sodium salicylate; E_M = molar extinction coefficient; V = total volume of the solution after adding trichloracetic acid; v = volume of serum used. In the usual case of 0.5 ml of serum diluted to 5 ml with trichloracetic acid the formula is as follows: Sodium salicylate mg/100 ml serum $= \frac{(O.D.) \times 10}{0.23}$.

This procedure was tested in guinea pigs which had received varying amounts of sodium salicylate. Table I shows the results of these determinations compared with the results obtained with a colorimetric method using the Folin-Ciocalteu reagent (3). The comparison is not based on duplicate determinations but on blood samples from different animals. The agreement, however, is satisfactory.

Ultraviolet absorption by salicyl derivatives. To test the specificity of the method described a number of substances related to salicylic acid were examined in the ultraviolet spectrophotometer. Acetylsalicylic acid has no absorption peak between 220 and 350 mμ. This substance cannot therefore be detected by the present method until it is hydrolyzed to salicylic acid (7). A metabolite of salicylic acid, salicyluric acid, has an absorption peak at 298 mμ and $E_M^{298} = 3498$. No change was observed in the spectrum by shifting the pH from 6 to 1. Gentisic (2, 5, dihydroxybenzoic) acid has an absorption peak at 320 mμ when dissolved in alkaline solution. ($E_M^{320} = 4784$). When, however, gentisic acid is mixed with serum, the peak shifts to 330 mμ and the extinction coefficient increases considerably ($E_M^{330} = 9152$). This shift is due to the action of serum and the development of the secondary peak depends on the amount of serum added to the substance. It has been assumed that gentisic acid is converted into another substance as soon as it is injected (8). Another dihydroxybenzoic acid, γ-resorcylic (2, 6-dihydroxybenzoic) acid, recently tested therapeutically (9), behaves in a similar manner. Its alkaline solution has an absorption peak at 302.5 mμ ($E_M^{302.5} = 3550$) but when it is mixed with serum a secondary peak develops between 290 and 300 mμ which increases with time and with increasing concentrations of serum. At the same time the original peak decreases.

Discussion. For routine clinical use the present method is simpler and faster than any of the technics described in the literature and requires only the use of an ultraviolet spectrophotometer. For research work, particularly the study of the metabolism of salicylate and its derivatives, the ultraviolet method has the advantage of a higher specificity than the reactions used by other methods. These are

non-specific reactions of the phenolic group with ferric ion(1), the Folin-Ciocalteu reagent(3,6) and nitrates(4) or development of fluorescence in alkaline solution(5). Separation of certain derivatives has been achieved by extraction with organic solvents(1,2,5). All of these methods require several reagents and involve a number of steps. The ultraviolet method either by itself or in combination with a colorimetric method achieves a simple, direct separate determination of the various salicyl compounds that are likely to be present simultaneously in biological fluids.

Summary. A method is described for determination of salicylate blood levels, based on the absorption spectrum of salicylic acid in the ultraviolet range. The method is suitable both for routine clinical use and for research work.

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Received April 7, 1952. P.S.E.B.M., 1952, v80.

Effect of Epinephrine, Glucose and Certain Steroids on Fatal Convulsive Seizures in Mice.* (19518)

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This is one of a series of experiments in an attempt to define the cause of immediate death following convulsive seizures. In previous studies one of us reported that the incidence of susceptibility to sound-induced convulsive seizures was associated with age(1-3). This was established from a survey of various inbred mouse strains; the age level of maximum susceptibility varied with the strain. The experiments reported here represent attempts to locate, by preventive measures, the cause of lethal convulsion and its onset in the dilute brown, DBA, mouse strain. This closely inbred strain is characterized by its high seizure susceptibility (97% incidence) and high lethal seizures (80% incidence) at the age level of 28-34 days. The pattern of the seizure is similar in appearance to the grand mal epileptic seizure of children.

Since the blood sugar level is low in convulsive human subjects, and glucose is often administered for treatment, this substance was given to a group of lethal-seizure susceptible mice. Behavior disorder may well be dependent upon abnormalities of blood composition and the sympathoadrenal system may be associated with the blood composition, on this assumption injections of glucose and of epinephrine were tested.

Material and procedures. A group of highly "lethal-seizure-susceptible" mice of the dilute brown, inbred strain, DBA, was employed. Both sexes in equal numbers were used and the ages at the first injection was 28-32 days. A description of the physical characters of the strain and the method used for inducing seizures by the sound of an electric bell were given in a former paper(3). The intensity of the sound was approximately 91 decibels with a variation of about ±2 decibels in different parts of the apparatus. The animals were individually tested in a galvanized iron tub with the bell hanging inside its wall. In each group there were from 40-50 individuals. Controls genetically related to the experimental

* Supported in part by a grant from the U.S.P.H.S.
† Graduate Students, participants in the Summer Studies Program.

TABLE I. Protective Effects of Epinephrine and Glucose on Fatal Convulsive Seizures in DBA Mice.

Groups and No. of animals	Size of each dose	No. of inj.	% of mice showing seizures 4 hr after last inj.* 0	Non-fatal	Fatal	Total
Epinephrine						
A (49)	10 μg	1	90	0	10	10
B (39)	50 "	1	100†	0	0	0
Epinephrine-glucose (10%)						
C (46)	10 μg-1 cc	1	94†	4	2	6
D (43)	"	5‡	90†	0	10	10
Glucose alone						
E (50)	1 cc-10%	1	50§	25	25	50
F (50)	"	2‡	54§	15	31	46
G (50)	1 cc-5%	1	55	17	28	45
Controls						
Direct, genetically related (76)			4	17	79	96
Standard, for strain (1316)			3	17	80	97

* Optimum duration of protection occurred between 1-4 hr.
† Continued protection from seizures to 24 hr.
‡ Inj. once a day on successive days.
§ Continued protection to 10 hr.

TABLE II. Protective Effects of Certain Steroids on Fatal Convulsive Seizures in DBA Mice.

Groups and No. of animals	Size of each dose	No. of inj.	% of mice showing seizures 8 hr after last inj.§ 0	Non-fatal	Fatal	Total
Pregnenolone (50)	50 μg	10†	66	0	34	34
Testosterone (50)	50	6*	85	8	7	15
Estradiol (50)	25	10*	50	0	50	50
(48)	50	6*	71	14	15	29
Cortisone (10)	50	1	25‖	37	38	75
Cortin‡ (40)	.05 cc	6*	12	28	60	88
(20)	.5	1	30	30	40	70
Controls						
Direct, genetically related (64)			4	17	79	96
Standard, for strain (1316)			3	17	80	97

* Inj. once a day on successive days.
† Inj. twice (10 hr apart) a day on successive days.
‡ 50 dog units per cc.
§ All gave some protection to 24 hr except cortisone which lost its effect after 6 hr.
‖ 4 hr after inj.

groups were used, also the established standard performance of this strain reported(3) previously was used for comparison. Experimental and control animals were all treated alike as to food and environment. The epinephrine doses were made by dilution of Parke Davis 1:1000 adrenalin chloride with physiological salt solution. The glucose was given in 5% and 10% strength. The size of each dose and the number of injections for each group are given in Table I. The animals were tested for convulsive seizures 1, 4 and 8 hours after the last injection. In cases where the protection was still effective the animals were tested up to 20 hours and longer. Previous experiments(4,5) with 6n-propyl thiouracil, administered in the food of DBA mice showed that this drug was a protective agent against the onset of seizures. This suggested a possible endocrine relationship. On this basis a group of hormonal compounds were tested: 1) pregnenolone acetate (Natolone-National

Drug Co.), 2) testosterone (Ciba-Methandren), 3) estradiol (Ciba ethinyl estradiol), 4) cortisone-Kendall's compound E, 5) cortin, (Parke Davis, Eschatin). From these compounds specific dosage strengths were made and were administered by injection. Two-hundred and twenty mice of the DBA strain were employed in this hormonal series. The ages of mice tested were 28-32 days except for the second estradiol (6 injections) group which were 36-38 days of age. The amount of each dose and the number of injections for each group are given in Table II, together with the percent of the individuals in each category which showed: 1) non-seizures, 2) non-fatal seizures, 3) fatal-seizures, and 4) total seizure incidence.

Results. The records and data of the epinephrine and glucose treatments are given in Table I. Among the combinations tried, the best treatment, in preventing lethal seizures was a combination of epinephrine with glucose. Fatal seizures were reduced to 2% of the individuals as compared with the 80% in untreated controls. The percentage of the individuals *not* exhibiting the onset of seizures was 90-94% as compared with the 3-4% of the control groups. Although the dose of 50 μg of epinephrine alone proved 100% effective, the treatment seemed to leave the animals somewhat lethargic.

The results in testing the few hormonal compounds are shown in Table II. Of the 5 compounds tested, testosterone was most effective with estradiol and pregnenolone close seconds. Cortisone and cortin were least effective.

Discussion. The wide range of effectiveness in the treatment of convulsive seizures indicates that the *mechanism* involved in the causes of such behavior disorder, as seizure susceptibility, is a complicated one. The results of our tests have been interpreted tentatively as indicating that susceptibility to sound-induced lethal convulsive seizures, in the DBA inbred strain of mice, is associated with the blood sugar level and also with the endocrine mechanism. Epinephrine alone and with glucose was very beneficial and the combination of the two appeared to be the best sustained protective measure against, not only the incidence of lethal convulsive seizures, but also against the incidence of the onset of seizures.

The hormonal compounds all steroids showed beneficial effects in various degrees on the incidence of, not only fatal seizures, but also the incidence of non-fatal seizures. Testosterone reduced the fatal seizure incidence from the control 80% to 7%, estradiol reduced it to 15% and pregnenolone to 34%. Moreover the incidence of seizures as a whole was also reduced from the control 97% to 15% by testosterone, 29% by estradiol, 34% by pregnenolone, 70% by cortin and 75% by cortisone. Since the availability of cortisone, at the time, was very limited it may be that the optimum dosage was not hit upon. It appears from our epinephrine tests that the medulla of the adrenal gland has more direct effect on the incidence of seizures than its cortex. The fact that all these hormonal compounds have some protective effect against the onset of sound-induced seizures in susceptible animals suggests that the mechanism involved is nonspecific in its physiology.

In his experiments relating to adrenalin injections to seizure-susceptibility in rats O. L. Lacey(6) found that a dose of 0.025 mg of adrenalin (¼ cc of 1:10,000 adrenalin chloride) administered to rats of 200 g body weight at 2-day interval over a period of 16 days, did not appreciably affect seizure-susceptibility. The number of individuals used by Lacey were 10 experimental (6 albinos and 4 gray rats) and 10 controls. The ages of his animals were "one year minus 3-8 days at the beginning of the experiments". Lacey claims no definite conclusion from his data as to the effect of the adrenalin injections. However he found the adrenalin injected rats averaged a 60% susceptibility incidence while the controls had a higher incidence or 70%. Also the average time to onset of the seizures of the adrenalin injected albino rats was 22.5 seconds while the control's average was 13.3 seconds. Larger doses of adrenalin might have given more definite results. His work(7) on the dependence of behavior disorder in the rat upon blood composition indicated that seizure-susceptible animals show significantly higher serum protein and significantly greater group

variability in blood sugar than do the non-susceptible animals.

Ginsburg, B., *et al.*(8) in their experiments with glutamic acid on DBA mice, used for their placebo injections a 5% dextrose solution (0.1 cc per 10 g of body weight) and claimed not to have found any effect of the dextrose injections. The dosages employed in our experiments were from 8 to 16 times greater than those of Ginsburg, and it will be noted that the larger dose gave better protection.

It has been shown that the susceptibility to seizures of the DBA mouse is hereditary(9-13), and although its precise mode of inheritance is not absolutely clear, there is evidence that multiple factors are involved. Our results strongly suggest that the manifestation or expression or release of the interaction of the genes involved may be controlled by a series of physiological factors, such as metabolism, and blood sugar level along with an optimum balance of hormonal secretions. All this supports the idea that the endocrine glands do play a definite role in the expression of convulsive seizures.

Summary. In an attempt to locate the cause which brings on convulsive seizures, agents to prevent lethal seizures in the DBA mouse strain, such as glucose, epinephrine and several hormonal compounds, steroids, were used. The DBA inbred mouse strain is characterized by its extremely high incidence of "lethal-convulsive-seizures" during the adolescent period. Testing mice in this period with various compounds showed that the DBA mouse responds to injections of all of them in various degrees of protectiveness against, not only fatal seizures, but also on the total seizure incidence. The untreated related animals showed 96% seizure incidence, while the epinephrine-glucose treated showed 6% incidence. As to "lethal-seizures", epinephrine with glucose reduced the fatality to 2%, while the untreated related controls had a 79% fatality. The hormonal compounds, especially testosterone, pregnenolone, and estradiol were effective in reducing the total seizure incidence from 96% to 15% and the fatal seizure incidence from 79% to 0-2%. The findings of our investigation support the conclusion that the supposedly genetic mechanism controlling the susceptibility to seizures, responds to treatments of glucose and various hormonal compounds. This experimental control of its expression suggests a mechanism which is sensitively responsive to endocrine secretions, and their interrelationships.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Effect of Salicylic Acid and Similar Compounds on the Adrenal-Pituitary System.* (19519)

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The extensive use of salicylic acid (SAL) in the treatment of rheumatoid arthritis and rheumatic fever left the impression that this drug has some specific effect in addition to its analgesic and antipyretic action. However, no satisfactory explanation for these good clinical results has been offered. The beneficial effect of adrenocorticotropic hormone (ACTH) in arthritis provided a new hypothesis. This made it possible to reinvestigate the pharmacology of SAL with special emphasis on the effect on the adrenal-pituitary system. An indication that such a mechanism might be involved was found in a report by Blanchard *et al.*(1) that a new "anti-arthritic" drug, 3-hydroxyphenylcinchoninic acid, may produce a depletion of adrenal ascorbic acid. A few exploratory experiments suggested a similar action of SAL, aminopyrine and cinchophen.

In the present study, the effect of SAL and related compounds on the adrenal-pituitary system was investigated in detail. The main purpose was to determine the specificity of any SAL effect and thereby to obtain a better understanding of the mechanism of the action of this drug. The ascorbic acid content of the adrenals was used as indicator since, according to Sayers and co-workers(2), this component can serve as a reliable measure of cortical activity. It is known that "stress" of various types may affect adrenal ascorbic acid and the latter apparently varies also with the weight and age of the experimental animals and seems also to be subject to seasonal fluctuations. Therefore, it was necessary to devise a procedure which would permit a comparison of experiments made at different times and under different conditions.

Methods. Normal female Wistar rats weighing 80 to 150 g were used. In each experiment the weight range was kept within ±20 g. The diet of Rockland pellets was available at all times *ad libitum*. Hypophysectomized female rats of the Sprague-Dawley strain‡ were used within 48 hours after the operation. The drugs were injected subcutaneously as sodium salts in aqueous solution at 37°C. All dosage figures refer to the free acid. The volume of the injection was kept constant at 0.3 ml per 100 g of body weight while the concentration was varied. It is essential that a sufficient number of control animals, receiving an equal volume of isotonic saline solution, is included in each experiment. Two hours after the injection, the animals were anesthetized by intraperitoneal injection of a 6.5% solution of pentobarbital sodium and the adrenals were quickly removed and weighed. The pH of the solutions used varied from about 5 to 9. As a rule, no attempt was made to adjust the pH since exploratory experiments with SAL had indicated that this factor is of no particular importance. The analytical procedures were: adrenal ascorbic acid, Roe and Kuether(3); salicylic acid, Brodie *et al.*(4), and Saltzman (5); salicyluric acid, Smith *et al.*(6); gentisic acid, Gerald and Kagan(7).

Results. The basic observation showing the action of SAL (300 mg/kg) is indicated in Table I, which contains also the adrenal ascorbic acid values of the corresponding saline controls. These data which were obtained over a period of 14 months, proved conclusively that SAL causes a depletion of adrenal ascorbic acid. They also showed that the values for both groups may vary considerably and in an unpredictable manner. Since such variations make it difficult, if not impossible, to evaluate the experimental data obtained at different times and under different conditions,

* Preliminary report presented at meeting and *J. Pharm. Exp. Ther.*, December, 1951, v103.

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‡ These animals were obtained from Hormone Research Laboratory, Chicago, Ill.

TABLE I. Adrenal Ascorbic Acid of Normal Rats (Average Values with Stand. Error) 2 Hr after a Subcut. Inj. of 300 mg/kg of Salicylic Acid (10% Sol. as Sodium Salt) or Saline Sol. (Control Group).

Control group		Salicylate group		
			Adrenal ascorbic acid	
No. of animals	Adrenal ascorbic acid in mg %	No. of animals	In mg %	% of control group
6	363 ± 28	6	225 ± 15	62
5	385 ± 21	5	214 ± 16	55.6
8	353 ± 19	8	209 ± 14	59.2
7	381 ± 17	4	220 ± 12	57.8
4	438 ± 25	4	266 ± 24	60.8
7	434 ± 9	8	273 ± 22	62.9
8	469 ± 18	7	271 ± 26	57.8
12	389 ± 28	7	216 ± 14	55.6
11	445 ± 34	8	256 ± 15	57.6
9	396 ± 23	10	248 ± 23	62.6
3	415 ± 21	3	244 ± 17	58.8
13	459 ± 13	6	281 ± 10	61.2
			Avg	59.3 ± .9

TABLE II. Adrenal Ascorbic Acid of Normal and Hypophysectomized Rats 2 Hr after a Subcutaneous Injection of Varying Doses of Salicylic Acid (As the Sodium Salts).

Drug	Dose	No. of animals	Adren. ascorb. acid in % of saline animals	Salicylate blood levels in mg %
		Normal animals		
0		24	135	
Isotonic saline	3 ml/kg	87	100	
Salicylic acid	75 mg/kg	12	89	11
	150	36	73	26
	300	76	59	42
	600	12	49	72
		Hypophysectomized animals		
Isotonic saline	3 ml/kg	12	100	
Salicylic acid	300 ml/kg	12	101	45

a simple method of comparison was desirable. This variability can be eliminated almost completely by expressing the results for the SAL animals in per cent of those of the control animals (Column 5). Therefore, all results presented in this paper will be expressed in per cent of the corresponding saline controls. This does not represent the "normal" concentration, since saline injections alone will cause within 2 hours a loss of about 25% of the ascorbic acid. If one assigns to the saline controls a value of 100%, untreated "normal" animals show accordingly a value of 135%.

The question of specificity of SAL action was approached in a 3-fold manner: 1) the dose-effect relationship; 2) the effect of hypophysectomy, and 3) the effect of various compounds, structurally related to or different from SAL. The results with varying doses of SAL (Table II) indicated that the depletion of adrenal ascorbic acid is in proportion to the amount of drug administered. The SAL blood level showed almost exactly the same dose relationship. The latter observation explains the previously made statement that the pH of the drug solution is of no particular importance. Obviously, one would not expect that variations in pH would greatly affect the absorption of a water soluble drug from the site of a subcutaneous injection.

Hypophysectomy completely abolished the effect of SAL (Table II), thus indicating the essential role of the pituitary and, specifically ACTH in the mechanism of the action of this drug. This observation excluded any direct action of SAL on the adrenals, since removal of the pituitary does not prevent the response of these glands to ACTH(2).

TABLE III. Aliphatic Acids Which Did Not Affect the Adrenal Ascorbic Acid of Normal Rats. Dose and No. of animals ( ) as indicated.

Acid	mg/kg	
Acetic	150	(4)
Adipic	150	(4)
Ascorbic	300	(11)
Citric	300	(12)
Dehydroascorbic	300	(4)
Fumaric	300	(7)
Gluconic	300	(4)
Glucuronic	300	(8)
Lactic	150	(7)
Pantothenic*	300	(9)
Pyruvic	300	(6)
Succinic	300	(8)

* Used as calcium salt.

The requirement of an intact pituitary and the good dose response relationship is only partial proof for the specificity of the SAL action. It is known that chemical, as well as physical, physiological, and even psychological stimuli, may influence directly or indirectly the adrenal function in the normal animal. As a more direct experimental approach to prove the specificity of action, the influence of varying the structure of the administered drug was studied. A total of 32 aliphatic and aromatic acids were used as sodium salts.

The results with 12 aliphatic acids were uniformly negative. With the exception of adipic acid, all substances investigated are involved in normal metabolic processes and 3 of the compounds (glucuronic, succinic and ascorbic acid) have been reported as being beneficial in the treatment of arthritis, although the opinion on this point is divided. The experiments with aliphatic acids also indicated that neither hypertonicity nor sodium content, which in several instances were considerably greater than those of saline solution, are of importance in this test.

The series of 20 aromatic acids is best divided into substituted benzoic and substituted salicylic acid (Table IV). In the first group only 2 compounds (benzoic and p-methoxybenzoic acid) produced a significant reduction in ascorbic acid. The observation of Blanchard that p-hydroxybenzoic acid was ineffective, was confirmed and extended to another salicylic acid isomer, m-hydroxybenzoic acid.

In the group of 11 substituted salicylic acids, 9 produced, like SAL, a depletion of ascorbic acid. The 2 exceptions were p-hydroxysalicylic and p-aminosalicylic acid, which on repeated tests gave negative results.

The figures in the fourth column of Table IV estimate the relative activity of substituted salicylic acids. The main variants were salicyluric and gentisic acid, both of which were much weaker than SAL, while benzoic and γ-resorcylic acid occupied an intermediate position. The 2 chlorosalicylic acids were more toxic than the other drugs because on a dose of 300 mg/kg all animals died before the end of the experiment. Whether or not this higher toxicity is the reason for the relatively great reduction of ascorbic acid, particularly after 4-chlorosalicylic acid, or whether it is a case of greater activity will require additional experiments.

Salicylaldehyde and salicylamide have a different structure and, strictly speaking, do not belong in a group of substituted salicylic acids. Qualitatively, they acted like SAL although their potency seemed to be less. This may be due to lower solubility which made it necessary to inject both compounds as aqueous suspensions.

The reason for the relatively low activity of salicyluric and gentisic acid was investigated in some detail, because both are normal metabolites of SAL in man and the latter is also used clinically. Qualitative tests for SAL in both blood and urine were negative, so that the effect could not be ascribed to partial hydrolysis or reduction of the administered drug. However, it was found that both salicyluric and gentisic acid were excreted very rapidly. The former was barely detectable 105 minutes after the injection and the latter was found in a concentration of only about 15 mg % 2 hours after a dose of 300 mg/kg. This rapid excretion of both salicyluric and gentisic acid may explain why considerably larger doses of these drugs were required to produce significant depletion of ascorbic acid.

Discussion. The experiments furnish adequate evidence that SAL reduces the adrenal ascorbic acid and that an intact pituitary is required to produce this effect. Furthermore, the results with the 32 different aliphatic and aromatic acids demonstrate that this ascorbic acid depletion is not due to general "stress,"

TABLE IV. Effect of Various Aromatic Acids§ on the Adrenal Ascorbic Acid of Normal Rats.

Drug	No. of animals	Dose, mg/kg	Ascorbic acid, % of control	Disassociation constant
Benzoic acid	11		72*	6.16×10^{-4}
m-HO-Benzoic acid	5		91	7.61
p-HO	5		115	2.80
o-CH_3O	13	300	101	8.3
p-CH_3O	23		79*	3.1×10^{-5}
o-C_2H_5O	5		106	7
p-C_2H_5O	9		90	5
p-NH_2	17		105	1.15
Salicylic acid	60	300	59*	1.06×10^{-3}
4-HO-Salicylic acid	11	300	106	5.05×10^{-4}
5-HO-Salicylic acid (Gentisic acid)	10	600	75*	1.06×10^{-3}
6-HO-Salicylic acid (γ-Resorcylic acid)‖	14		72*	5×10^{-2}
5-CH_3O-Salicylic acid	6	300	69*	
3-CH_3	6		65*	1×10^{-3}
4-CH_3	6		69*	
4-Cl	10	150	63*	
5-Cl	10	150	69*	1.95×10^{-3}
5-HO_3S	6	300	64*	
4-NH_2	12	300	109	3.5×10^{-7}
Salicyluric acid‖	17	426‡	82†	
Salicylaldehyde	6	300	71*	
Salicylamide	6	300	73*	

* Statistically significant difference ($p = .01$ or less).

† $p = .02$.

‡ This quantity of salicyluric acid is equimolar to 300 mg of salicylic acid.

§ The authors are indebted to Dr. T. R. Aalto of Heyden Chemical Corp. for samples of many of the substituted benzoic and salicylic acids.

‖ Salicyluric acid and γ-resorcylic acid were prepared by N. H. Leake and M. L. Fielden of the Research Department of S. E. Massengill Co.

because many of the compounds tested did not produce a greater change than saline solution. The results with molecular variants are interpreted as indicating a specific effect of substances with a characteristic chemical structure. On the basis of the available evidence, the salicylic acid configuration represents one example of such structure.

Since the ascorbic acid content of the adrenals is considered a reliable indicator of cortical activity, which in turn is governed by ACTH, all the experimental observations fit into the assumption that SAL stimulates through some specific mechanism the output of ACTH by the pituitary. At present, it is impossible to answer the following questions: 1) Is the observed effect the result of increased production or of mobilization of a stored reserve of ACTH? 2) Is the pituitary the primary target of the action of SAL or is it only secondarily stimulated following a primary effect on some other receptor such as the hypothalamus? It may be mentioned that more recent experiments with animals under complete pentobarbital anesthesia indicate quite conclusively that the hypothalamus is directly involved in the SAL action. The details of this work will be presented elsewhere.

As far as specificity is concerned, it is not restricted to the SAL configuration, but includes, at the present time, benzoic acid and, as shown by Blanchard *et al.*(1), also aminopyrine, cinchophen and certain cinchoninic acid derivatives. It is of interest that all cinchoninic acid derivatives found to produce ascorbic acid depletion have an hydroxylgroup in ortho position to a carboxylgroup. It is noteworthy that all the above mentioned drugs have been or are used in the treatment of arthritic conditions.

In a recent publication, Reid *et al.*(8) have suggested that the action of SAL is connected with its ability to form chelate compounds. In support of this theory, these authors claim

that γ-resorcylic acid (2,6-dihydroxybenzoic acid) which has a stronger chelation effect than SAL, is more effective in the treatment of rheumatic disease. In the ascorbic acid depletion tests, reported in this study, γ-resorcylic acid is definitely less active than SAL. This agrees with a short statement in Reid's paper. Since the disassociation constant of organic acids is considered to reflect their chelation ability, the pK values, as reported in the literature, are listed in Table IV. It is apparent that the available figures do not indicate a relationship between the disassociation constant of these drugs and their effect on adrenal ascorbic acid.

Summary. 1. The effect of salicylic acid and similar compounds on the adrenal-pituitary system has been investigated. 2. Salicylic acid and a number of substituted salicylic acids cause a significant depletion of adrenal ascorbic acid. The only exceptions found so far are p-aminosalicylic and p-hydroxysalicylic acid. 3. The SAL blood levels parallel very closely the ascorbic acid depletion of the adrenal. 4. Hypophysectomy prevents the effect of salicylic acid on adrenal ascorbic acid. 5. Benzoic acid has an effect similar to that of salicylic acid, while several substituted benzoic acids, including p-aminobenzoic acid, are ineffective. 6. Twelve aliphatic acids of various types did not produce a change in adrenal ascorbic acid. 7. The experimental results are interpreted as indicating a specific direct or indirect action of salicylic acid on the anterior pituitary which results in the production or release of increased amounts of adrenocorticotropic hormone.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Studies on Vi Antigen. I. Relative Vi Antigen Content of V Form Cultures. (19520)

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It has been demonstrated that vaccines prepared from rough Vi strains of *Salmonella typhosa*(1,2), and from Vi strains of *Paracolobactrum ballerup*(3,4) and *Escherichia coli*(4), none of which contain typhoid somatic components, are effective in the immunization of mice against infection with Vi + O strains of *S. typhosa*. Although 4 bacterial species are known to possess Vi antigen, information concerning their content of this component was lacking. The object of this investigation was to compare, by mouse protective potency tests, the Vi antigen content of these bacterial species to determine which provided the richest source of this antigen.

Cultures. The V form *S. typhosa* strains 58 and Ty2 were the challenge organisms used in the mouse protection tests. Strain 58 exhibited evidence of agglutinability by typhoid O antiserum, while Ty2 was quite resistant to O agglutination. When administered intraperitoneally in mucin, both strains exhibited maximum mouse virulence ($LD_{50} < 10$ organisms). When injected in saline the LD_{50} for strain 58 was approximately 220 million, while that for Ty2 was approximately 40 million. The other V form cultures used in this study were the Kauffmann strains *E. coli* 5396/38, *P. ballerup* 7851/39 and *S. paratyphi* C (East Africa). The properties of

these cultures have been described by Kauffmann(5). The only known antigenic component common to these bacterial species is the Vi antigen. Cultures were taken from the lyophilized state and maintained in the V form on nutrient agar (pH 7.6) by routine plating and colony selection. *E. coli* and *P. ballerup* cultures were incubated at 25°C, *S. paratyphi* C and *S. typhosa* strains at 37°C. Colonial morphology was examined with the aid of oblique illumination(6) and antigenic composition of each strain was checked periodically by tube agglutination tests with specific O and Vi antisera.

Mass culture production. Acetone killed and dried organisms were prepared as a convenient and stable source of Vi antigen since it had been reported(7) that V form *S. typhosa* prepared in this manner stimulated the production of Vi antibody in animals as readily as viable organisms. Prior to the mass culture of a given V form strain, the culture was plated, typical smooth dense colonies were selected and antigenic composition was tested until the strain was considered to be in the desired condition with respect to Vi antigen. Repeated colony selection was essential for the maintenance of the V forms of these strains. For large-scale production runs the organisms were cultured on veal infusion agar (pH 7.6) in Kolle flasks, from 18 hour seed agar cultures suspended in veal infusion broth, and incubated for 18 hours at the temperature appropriate for the species. Each step of the operation was checked by plating, examination of colonial morphology, microscopic examination of Gram stained preparations and agglutination tests with O and Vi antisera. When the control plates at the start and end of the production run revealed 95-99% V form colonies, this was taken as evidence that the harvest from the Kolle flasks likewise consisted of V form organisms in the same proportions. The growth was harvested in distilled water (10 ml per Kolle flask) and the organisms were precipitated from suspension with three volumes of acetone and collected by centrifugation. The organisms were then resuspended in fresh acetone and held at 37°C for 24 hours. Culture at this stage indicated that no viable organisms remained. The bacterial precipitate was collected on fritted glass filters. Final traces of acetone and moisture were removed by desiccation *in vacuo*. The total yield from 360 Kolle flasks was 60 to 90 g of dry organisms.

Vi antigen extracts. HCl extracts of the acetone dried organisms were obtained by the procedure employed by Luippold(8). Removal of the Vi antigen from the bacterial mass was facilitated by frequent shaking at room temperature for 24 hours. The resultant turbid suspension was cleared by centrifugation and then adjusted to pH 7.

Mouse protective potency tests. The assay procedure employed was an active-immunization mouse protection potency test based on the use of graded immunizing doses and a constant challenge dose(9). White Swiss mice, Bagg strain, weighing 14 to 16 g, and segregated as to sex, were assigned to jars in groups of 5. Assignment of animals to jars, assignment of jars to locations in the animal room, and the order of immunization and challenge injections, by groups, were all determined by randomization procedures. All immunizing injections were given intraperitoneally in a standard volume of 0.5 ml. Physiological saline was used as diluent. Four groups of 5 mice each, 2 groups of females and 2 groups of males, were employed for each dilution level tested. In individual assays, 3 or more levels of 4- or 5-fold progressive dilutions were employed. The mice were challenged 6 days after the immunization by the intraperitoneal route. Two types of challenge suspension were employed, 1000 *S. typhosa* 58 suspended in 5% gastric mucin, and 100 million *S. typhosa* Ty2 suspended in saline. The period of observation following challenge was 3 days.

Since the assay procedure was based on protection against typhoid challenge, and since the comparison of the Vi content of the various V form organisms with that of *S. typhosa* was the factor of prime interest, acetone dried strain Ty2 (Table I) and an HCl extract of strain 58 (Table II) were adopted as reference standards for comparison of the 3 sources of Vi antigen. The method of Knudsen and Curtis(10) which involves transformation of the survival data to angle values was employed to calculate the ED_{50} and relative potencies of these preparations.

TABLE I. Survival of Mice Immunized with Acetone Killed and Dried V Form Cultures and Challenged with *S. typhosa* Ty2.*

V form culture	Acetone dried organisms, immunizing dose (μg)						E.D.$_{50}$ (μg)	Relative potency (*S. typhosa*=1)
E. coli 5396/38	.02	14/20†	.004	9/19	.0008	4/20	.0094	6
P. ballerup 7851/39	.10	17/20	.02	11/20	.004	5/20	.0149	3.2
S. paratyphi C (East Africa)	2.5	8/20	.5	5/20	.1	1/20	4.340	.02
S. typhosa Ty2	.5	18/20	.1	15/20	.02	5/20	.0504	1

* Challenge dose, 140000000 organisms suspended in .5 ml of saline inj. intraper.
† Numerators denote No. of survivors at 72 hr; denominators, total mice tested.

TABLE II. Survival of Mice Immunized with HCl Extracts of V Form Cultures and Challenged with *S. typhosa* 58.*

V form culture	1% HCl extract, immunizing dose (μg)†						E.D.$_{50}$ (μg)	Relative potency (*S. typhosa*=1)
E. coli 5396/38	.120	19/20‡	.030	11/20	.0075	4/20	.022	12
P. ballerup 7851/39	.299	18/20	.075	14/20	.019	4/20	.049	5.5
S. paratyphi C (East Africa)	51.5	18/20	5.15	8/20	.515	2/20	6.100	.04
S. typhosa 58	1.65	17/20	.165	8/20	.0165	1/20	.257	1

* Challenge dose, 1000 organisms suspended in .5 ml of 5% mucin, inj. intraper.
† Extracts tested in the fluid state. Solids determined by drying extracts at 90°C to constant wt.
‡ Numerators denote number of survivors at 72 hr; denominators, total mice tested.

Results. The results of mouse protective potency tests on animals immunized with acetone killed and dried V form coli, ballerup, typhoid, paratyphi C and with HCl extracts of these organisms are detailed in Tables I and II. These tests were repeated on several occasions with similar results. In each experiment the four cultures were assayed simultaneously in order that their protective potencies might be compared directly.

The data given in Table I show that acetone dried *E. coli* was the best protective antigen. In terms of relative potency it possessed twice the activity of *P. ballerup* and six times that of *S. typhosa* Ty2. In contrast, *S. paratyphi* C showed only a trace of activity. The data in Table II indicate that the *E. coli* extract with a relative potency of 12 was the most active antigen, being approximately twice as potent as the *P. ballerup* extract and twelve times as active as the extract from *S. typhosa* 58. *S. paratyphi* C possessed minimal activity. The relative potencies of the four extracts corresponded reasonably well to the values obtained with the intact organisms which suggests that extraction of Vi antigen from these organisms was fairly uniform.

The protection against typhoid challenge afforded by these organisms and extracts was considered to be related to the quantity of Vi antigen contained in the immunizing dose. It was assumed that the observed differences were a reflection of quantitative differences in Vi antigen content of these strains. The possibility remains that there may be qualitative differences as well. However, no evidence to support the latter possibility was obtained in this study.

These findings in addition to providing confirmation of the effectiveness of *E. coli* 5396/38 organisms and extracts in protection of mice against typhoid challenge(4,8), show by direct comparison, in quantitative bioassays, the extent to which this strain of *E. coli* surpasses *S. typhosa* and the other V form species as a source of Vi antigen for the purposes of concentration and isolation.

Summary. The known V form non-typhoid organisms, *E. coli, P. ballerup* and *S. paratyphi* C in the form of acetone dried organisms or HCl extracts are all effective in the immunization of mice against typhoid challenge. Their relative potencies in this respect, compared with an arbitrarily fixed value of 1

for acetone dried *S. typhosa* Ty2 are as follows: *E. coli*, 6; *P. ballerup*, 3; *S. paratyphi* C, 0.02. It thus appears that *E. coli* 5396/38 is the richest source of Vi antigen among the known V form *enterobacteriaceae*.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Failure to Demonstrate *in Vitro* Lysis of Sensitized Guinea Pig Leucocytes by Dog Hemoglobin Antigen.* (19521)

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Squier and Lee(1) have reported demonstration of a mean average reduction of 43% in the total number of leucocytes resulting from the addition of short ragweed antigen to heparinized whole blood from patients sensitive clinically and by skin test to the ragweed pollen. This reduction affected chiefly the polymorphonuclear leucocytes. It was postulated that the leucocyte reduction was due to an antigen-antibody reaction. It was further postulated that the antibody in this instance was a thermolabile reagin inactivated by heating to 56°C. Franklin and Lowell have failed to duplicate these results(2). This report describes an attempt to apply Squier and Lee's technic to a study of some phases of hypersensitivity and anaphylactic shock, using mature guinea pigs.†

Method. The hemoglobin suspension utilized as antigen in this experiment was prepared from citrated whole blood of dogs. The erythrocytes were separated by centrifugation and washed 3 times with normal saline, following which they were lysed with distilled water. Sufficient 18% NaCl was added to make a slightly hypertonic solution and again centrifuged to precipitate the stroma of the erythrocytes. The supernatant was drawn off and to this added about ⅓ its volume of "Amphogel". After standing for about 2 hours this latter mixture was filtered through coarse filter paper. The filtrate contained nearly pure hemoglobin and was utilized as antigen in the experiment. Twelve guinea pigs ranging in weight from 300 to 400 g were sensitized by 2 subcutaneous injections of 0.2 ml portions of the hemoglobin antigen at 48-hour intervals. Ten control guinea pigs ranging in weight from 300 to 350 g were similarly injected with 0.2 ml portions of normal saline. In the experimental group 5 females and 7 males were used. In the control group 5 females and 5 males were used. These animals were caged in groups of 5 and the sexes kept separated. None of the females were known to be pregnant. The diet was considered adequate and all of the animals were apparently healthy. In the control group blood was drawn by intracardiac puncture on the 13th or the 15th day after the second subcutaneous dose of saline. In the experimental group blood was similarly drawn on the 20th, 21st, or 32nd day after the second sensitizing dose of hemoglobin. The experi-

* This study was carried out under contract with the Division of Biology and Medicine, U.S.A.E.C.

† Animals obtained from Carworth Farms.

mental animals were subsequently subjected to 1.0 ml intracardiac doses of the antigen to prove sensitivity. The blood removed from each guinea pig was heparinized and divided into equal portions in 2 Wassermann bottles. To the first portion was added normal saline and to the second hemoglobin antigen. The concentration of blood to antigen varied from 2:0.1 to 3:0.1 ml. In order to rule out the possibility of insufficient concentration of antigen, an additional 0.4 ml of antigen was added to the blood specimens from the last 2 animals after 120 minutes, increasing the concentration of blood:antigen in these instances to 2.6:0.5 ml. In each instance the ratio of blood:saline was the same as that of blood: antigen. The tubes were rotated vigorously to mix and white cell counts made immediately after mixing blood and saline or blood and antigen, and repeated at 30, 60, and 120 minutes from each tube. With the last 2 experimental animals counts were also made at 180 and 240 minutes during which time a significant change did not occur. In each instance 2 counts were made at each interval and the results recorded represent averages of these counts. Diluted blood specimens were counted within a few minutes of being drawn into the pipettes. The time interval between cardiac puncture and the addition of the saline or antigen to a given specimen was usually 1 to 2 hours. In the case of the last 2 experiments, however, the delay was 15 and 20 minutes respectively.

Results. Blood from control and sensitized animals showed no leucocyte lysis when tested *in vitro* with the hemoglobin antigen. The interval of time elapsing between sensitization and attempted *in vitro* shock had no detectable effect on results. Concentration of antigen in the range studied likewise did not significantly alter results. No sex differences were noted.

It was found that the mean white cell count of the sensitized guinea pigs made immediately after mixing in the tubes with blood and saline is 32.7% above that of the non-sensitized pigs. In the tubes with blood and hemoglobin antigen the mean white cell count of the sensitized guinea pigs is 34.0% above that of the non-sensitized pigs. It was not determined whether the higher white cell counts of the sensitized animals was due to the presence of eosinophils.

TABLE I. Total Leucocyte Counts on Heparinized Blood from 10 Control Guinea Pigs.

Sex	Blood + normal saline: Immed.	30″	60″	120″
♀	6500	6225	5950	5525
♀	5225	5650	5475	5425
♀	3450	3525	3150	2950
♀	7625	7525	6650	5975
♀	3350	3450	3825	3175
♂	3600	4200	3625	3825
♂	5600	5500	6050	5325
♂	4350	3950	3425	3950
♂	5000	5400	6150	5600
♂	5600	5975	5450	4550
Mean	5030	5140	4975	4630
	Blood + hemoglobin antigen			
♀	6675	5875	6925	5975
♀	4725	5325	5475	6075
♀	3725	3025	3200	3150
♀	6675	7050	6475	6175
♀	2725	3450	3550	3850
♂	2925	2500	3000	2975
♂	4925	4950	5225	5225
♂	4750	4600	3850	4250
♂	4475	6675	5600	4875
♂	5450	4375	5025	3225
Mean	4705	4783	4833	4578

Of the experimental animals in this series 2 died of hemorrhage incident to intracardiac puncture. Of the remaining 10 animals, fatal anaphylactic shock was induced in 7 on injection of 1.0 ml of hemoglobin antigen. There was mild shock in one animal and no shock was apparent in two. These results are tabulated in Tables I and II.

Discussion. The coefficient of variation for white cell counts made from a single blood specimen as reported by Berkson is 10.7% (3). A criticism of inadequate sampling with routine technic would therefore be valid. It is doubtful, however, that errors inherent in the counting technic could obscure a mean reduction of 43% in the total white cell counts were it present, or show one that is not present.

Seven of the 10 animals subjected to the shock dose of hemoglobin antigen died immediately of typical anaphylactic shock. This demonstrates that the majority of the experimental animals in this series were highly sensitive to the antigen employed.

The significance of the increased mean white cell count of the sensitized guinea pigs over

TABLE II. Total Leucocyte Counts on Heparinized Blood from 12 Guinea Pigs Sensitized with Hemoglobin Suspension.

Sex	Days inc. (*In vitro* shock)	Blood + normal saline				Blood + hemoglobin antigen				Days inc. (*In vivo* shock)	Result shock dose
		Immed.	30″	60″	120″	Immed.	30″	60″	120″		
		3650	3100	2800	2975	2525	3275	3250	2575	Acc. hemorrhage	
		6325	5725	7200	6275	5450	5350	5250	5300		
♀	20	3225	3525	4375	3700	3125	2925	3425	3300		
		6325	6075	6625	6475	6075	5025	5600	5575	25	Fatal
		10550	8400	9550	9375	10200	9425	8475	8625		
		7675	8000	7650	8325	8350	7675	7350	8225		
		8425	7125	7600	7400	8350	7975	7425	8000		
♂	21	8500	7325	6250	6475	7375	7100	7225	6700	25	No shock
		5550	5375	6200	6525	5175	5725	6250	5950		
		8450	9700	9125	10325	8225	9150	9150	9275	Acc. hemorrhage	
♂*(1)	32	5825	6275	5525	6500	5550	4950	5950	5550	32	Fatal
♂*(2)	32	5575	4825	5500	5025	5275	5150	4825	4525	32	Mild shock
	Mean	6673	6288	6536	6615	6306	6144	6181	6133		

* Last 2 guinea pigs counted 2 additional hr. (Additional .4 ml saline and antigen added to respective tubes.) Values follow:

	Blood + normal saline		Blood + hemoglobin antigen	
	180″	240″	180″	240″
(1)	5025	4975	4325	4700
(2)	4625	4525	4150	4150

the non-sensitized pigs as related to leucocyte lysis in shock is not clear.

Summary and conclusions. Twelve guinea pigs were sensitized with dog hemoglobin. Subsequently blood specimens were taken and heparinized and a portion of these specimens mixed with the antigen. White cell counts were done immediately after mixing, and repeated at 30, 60, and 120 minutes. In 2 cases counts were also made at 180 and 240 minutes. Controls of blood and saline in the same ratio as blood and antigen were counted concomitantly on each guinea pig. Blood specimens from 10 non-sensitized guinea pigs were similarly collected and counted. No significant difference in the total white cell counts from sensitized or non-sensitized animals either when mixed with saline or with the hemoglobin antigen was detected. The total mean white cell count of the sensitized animals in this series was 32.7% and 34.0% above that of the non-sensitized animals in the control and experimental tubes respectively. The above findings indicate that *in vitro* lysis of sensitized guinea pig leucocytes by dog hemoglobin antigen does not occur.

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Received January 24, 1952. P.S.E.B.M., 1952, v80.

Effect of Electroconvulsive Shock on Adrenal Cortex of the Rat.* (19522)

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In accounting for the effects of electroconvulsive shock in disrupting the maternal be-

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havior of white rats, Rosvold(1) proposed that many of the behavioral disturbances arise directly or indirectly from endocrine disorders brought on by electroshocks. That some endocrines actually are affected was demonstrated by Bacon and Rosvold(2) who found abnormalities in the pituitary and ovaries following a series of electroconvulsions. The changes in the pituitary suggest that the adrenal gland also might be affected. If the behavioral disturbances are related to endocrine dysfunction(1), these changes in the adrenal should occur under conditions of electroshock similar to those used in the behavior studies. Experiment I tests this hypothesis.

Porter and Stone(3), Siegel, McGinnies, and Box(4); Stone and Walker(5), demonstrated that some of the disruptive effects of electroconvulsions on the rat's behavior can be avoided by anesthesia of the rat during shock. The proposition that the disturbances in behavior are directly related to endocrine dysfunction would be strengthened if the adrenal changes were avoided under conditions of electroshock in which the behavioral effects did not occur. Experiment II tests this hypothesis.

Exp. I. *Animals.* Thirty-one adult male and 19 adult female albino rats of the Sprague-Dawley strain similar in body weight were housed in individual cages in a room in which the temperature was approximately 80°. They were maintained on laboratory fox chow and water *ad libitum.*

Shock procedure. The electroshock equipment was similar to that designed by Hayes (6) and used in the Stanford and McGill laboratories in studies of the effects of electroconvulsive shock on learning, maternal behavior, and pregnancy. It was powered by 110 volts A.C. and consisted essentially of a transformer delivering a 1500 volt secondary current, a 200 millisecond timer, and a 26,000 ohm resistor in series with the rat. The rat received approximately 50 ma for 0.2 second through alligator clips fastened to the ears. These conditions invariably produced in the normal rat a generalized convulsion of the type described as tonic-clonic by Golub and Morgan(7). One group of 15 male and one of 10 female rats received electroconvulsive shocks through clip electrodes fastened to the ears. A control group of 16 males and one of 9 females had the electrodes clipped to the ears, but no current was delivered. Each of the shock groups received shock once per day for 10 days. Twenty-four hours after the last electroconvulsive shock, all the animals were anesthetized with nembutal and the adrenal glands removed, cleaned and weighed. The adrenals of 6 controls and 6 shocked animals were placed in metaphosphoric acid for determination of ascorbic acid content.

TABLE I. Mean Wt (in mg) of Adrenals of Shock and Control Groups.

Group	No. of animals	Means	$S.E_M$	P value of M. diff.
1. Shock	15 ♂	25.9	1.61	<.01
2. No shock	16 ♂	20.1	.55	
3. Shock	10 ♀	34.2	1.09	<.01
4. No shock	9 ♀	29.2	.64	
5. Shock	10 ♂	26.7	1.19	<.01
6. Shock and ether	8 ♂	19.6	.73	
7. Shock	10 ♂	26.7	1.19	<.02
8. Shock and nembutal	9 ♂	23.2	1.04	
9. Ether	8 ♂	20.2	.78	>.05
10. Shock and ether	8 ♂	19.6	.73	
11. Nembutal	9 ♂	23.2	1.25	>.05
12. Shock and nembutal	9 ♂	23.2	1.04	

Results. Table I (rows 1-4) shows the comparison of adrenal weights of control groups and shock groups. It is clear that a series of electroconvulsive shocks results in hypertrophy of the adrenal glands in both sexes. Since the milligram per cent of ascorbic acid in the adrenals of the shocked rats is not significantly different from that of the control ($t = .14$ $p > .05$), it may be that the hypertrophy is associated with increased secretory function in the manner described by Sayers(8) for his type 2 stress.

Exp. II. *Animals and procedures.* Forty-four male adult albino rats of the Sprague-Dawley strain similar in body weight were maintained and, when indicated, shocked as described in Exp. I. Eight received shock during ether anesthesia and nine during nembutal anesthesia. Ten controls received shock without anesthesia; while 8 controls were

anesthetized with ether but not shocked, and 9 anesthetized with nembutal but not shocked. Ether was administered to each rat individually by inserting the rat's head in a small bottle containing cotton saturated with ether. Nembutal was administered by intraperitoneal injection in about .3 cc doses calculated to contain 4.5 mg of nembutal per 100 g of body weight. Surgical anesthesia was always achieved before the rat was shocked. Such rats behaved like those described by Porter and Stone(3) in that the generalized epileptiform convulsion was absent, and the post convulsive period was more like rats recovering from anesthesia than shock. Twenty-four hours after the last shock in a series of 10, all the rats were anesthetized with nembutal and the adrenals removed, cleaned and weighed.

Results. Table I (rows 5-8) contains the data for a comparison of adrenal weights of shocked animals with those shocked during anesthesia. The adrenals of animals receiving electroshocks are significantly larger than those shocked during ether or nembutal anesthesia. The data in rows 9-12 of Table I indicate that there is no reliable difference in the size of the adrenals between those animals shocked under anesthesia, and the controls anesthetized but not shocked, demonstrating that the differences shown in rows 5-8 are not an artifact of the effect of anesthesia on the adrenals.

Conclusion and discussion. An effect of a series of 10 electroshocks is to increase the size of the rat's adrenals. This effect is inhibited if the shocks are administered during anesthesia of the rat. Since the behavioral effects of electroconvulsive shock occur or are inhibited under circumstances in which the effects of electroshocks on the adrenal cortex occur or are avoided it is reasonable to assume that in the rat the behavioral and endocrine effects are related. That the same relationship between the adrenal cortex and behavior may hold for humans is suggested in studies by Hoagland *et al.*(9,10), and recently by Graham and Cleghorn(11) who demonstrated that the clinical condition of psychotic patients during a course of electroshock therapy may be reflected in the function of the adrenal cortex.

Summary. Hypertrophy of the rat's adrenal glands has been shown to follow a series of electroconvulsive shocks administered under conditions known to disturb behavior. The adrenal enlargement was avoided by administering the series of shocks during anesthesia of the rat, a circumstance known to inhibit the behavioral disturbances following a series of electroconvulsive shocks. The coincident changes in adrenal size and in behavior suggest that the endocrine changes may mediate the effects of electroconvulsive shocks on the behavior of the rat.

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Received March 10, 1952. P.S.E.B.M., 1952, v80.

Failure of Two Strains of *Endamoeba histolytica* to Develop Resistance To Amoebacidal Agents *In Vitro*. (19523)

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A point of critical importance in the use of antibiotics and other compounds in the treatment of infectious diseases is the ability of the infectious organism to develop resistance to the therapeutic agent. The tendency of certain susceptible bacterial strains to become resistant to streptomycin, sulfonamides, and certain other commonly used drugs is well known. So far, little is known about the ability of *Endamoeba histolytica* to develop such resistance.

This brief report will describe attempts to demonstrate the development of resistance to aureomycin, terramycin and emetine HCl by two strains of *Endamoeba histolytica*.

Material and methods. The Luna† and 200‡ strains of *Endamoeba histolytica* were used in the experiments to be described and the substrate was the Shaffer-Frye (S-F) medium(1). The *S-F medium* was made up in the usual manner and sufficient aureomycin, terramycin, or emetine HCl added to bring the final concentration to the proper level. It was desirable to use a concentration sufficiently high to markedly inhibit propagation of the amoebae, but still allow enough propagation to be able to make serial transplants. The amounts used were arrived at by a series of trials in which serial transplants were attempted in the presence of varying concentrations of the drugs to be used. It was found that the desirable concentrations were, aureomycin 0.013 mg/ml, terramycin 0.04 mg/ml, and emetine HCl 0.0072 mg/ml of completed medium. These amounts allowed but minimal propagation of the amoebae, but it was possible to maintain serial 48- to 72-hour transplants of the two strains. In terms of actual numbers, the amoebae propagated to 1/8 to 1/4 the numbers usually seen in S-F medium without drugs. After 10, 20, 30 and 38 transplants, drug sensitivity tests were done on the two strains being studied using the method of Biegeleisen and Shaffer(2). Simultaneous tests were done on the normal Luna and 200 strains and the results compared. In preparation for the *sensitivity tests*, the amoebae were transplanted from the drug-containing medium to routine S-F medium for one transplant. From this transplant inoculations were made into 7 tubes of S-F medium and these cultures were incubated at 37°C. A similar series of tubes was inoculated with "normal" amoebae. At the end of 48 hours the cultures were examined for amoebae and the readings recorded. Serial 2-fold dilutions of the drugs were then added in a volume of 1 ml to the first 6 tubes, and 1 ml of saline (0.85% NaCl) was added to the seventh tube as a control. The cultures were reincubated at 37°C and examined after 24 and 48 hours. The relative numbers of amoebae remaining at the time of examination were recorded.

Periodically, during the course of the 38 transplants, attempts were made to increase the concentrations of the drugs. This was done by adding extra culture tubes containing more drug and attempting to make serial transplants. In no case was this successful, even small increases in the drug concentrations resulted in loss of the transplants after 1 or 2 transfers.

Results. The results of the drug sensitivity tests done on the experimental and "normal" *E. histolytica* of the Luna and 200 strains are shown in Table I.

Examination of these results reveals that

* Aided by a grant from the Institute for Microbiology, U. S. P. H. S.

† The Luna strain of *Endamoeba histolytica* was isolated by J. G. Shaffer in 1948 from a patient with acute amoebic dysentery directly from feces into S-F medium. It has never been propagated in any other medium.

‡ The 200 strain of *Endamoeba histolytica* was obtained in 1950 from Mr. M. C. McCowen of the Lilly Research Laboratories. It was obtained by Mr. McCowen from Dr. C. W. Rees of the National Institute of Health.

TABLE I. Results of Drug Sensitivity Tests on the Luna and 200 Strains of *E. histolytica* Transplanted in Presence of Low Concentrations of Aureomycin, Terramycin, and Emetine HCl Compared with Sensitivity of Unexposed Amoebae of the Same Strains.

	Sensitivity											
	Luna strain						200 strain					
	Aureomycin* (mg/ml)		Terramycin (mg/ml)		Emetine HCl (mg/ml)		Aureomycin (mg/ml)		Terramycin (mg/ml)		Emetine HCl (mg/ml)	
Transplant	N.†	E.‡	N.	E.	N.	E.	N.	E.	N.	E.	N.	E.
10	.0332	.0332	.1329	.1329	.0216	.0216	.0332	.0332	.1329	.1329	.0216	.0432
20	.0332	.0166	.2658	.2658	.0432	.0432	.0166	.0332	.1329	.1329	.0216	.0216
30	.0664	.0332	.1329	.1329	.0108	.0108	.0332	.0166	.0664	.0329	.0108	.0216
38	.0332	.0332	.2658	.2658	.0216	.0216	.0166	.0166	.1329	.1329	.0216	.0432

* Concentrations in these columns resulted in a decrease of at least 75% in the number of amoebae seen in cultures after 48 hr.

† "Normal" *E. histolytica* not exposed to the drugs.

‡ Experimental *E. histolytica* transplanted in the presence of partially effective concentrations of the drugs.

in no case was there more than a 2-fold difference in the concentration of a compound found capable of destroying 75% or more of the "normal" or experimental cultures. In 5 instances the concentration found effective in the experimental cultures was twice that found effective in the "normal" cultures, in 3 instances the reverse was true, and in 15 instances the concentrations were identical. Thus, the differences observed were probably due to experimental error and no evidence of the development of resistance by either strain of *E. histolytica* was observed. The inability to maintain transplants in the presence of drug concentrations somewhat above those routinely used, as mentioned previously, is also interpreted as evidence of failure to develop resistance.

Discussion. The method used in these experiments for testing the drug sensitivity of *E. histolytica* has the disadvantage that there are still a certain number of viable bacterial cells in the cultures at the time the compounds are added(2). However, evidence has been obtained(3) which indicates that the effects observed using this technic are the result of activity of the drugs on the amoebae rather than the bacteria. If this be true, then the results shown in the table are suggestive that *E. histolytica* does not readily develop resistance to aureomycin, terramycin, or emetine HCl *in vitro*.

Summary. Two strains of *E. histolytica* failed to show evidence of the development of resistance to aureomycin, terramycin or emetine HCl after 38 serial transfers in S-F medium containing partially effective concentrations of the agents.

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Received March 17, 1952. P.S.E.B.M., 1952, v80.

Viability and Dispersion of BCG Inoculated Subcutaneously in Guinea Pigs. (19524)

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Studies on the fate of BCG in guinea pigs inoculated by the intradermal route(1), multiple puncture(2,3), and by scarification(4,5) have shown that BCG were dispersed to regional lymph nodes within one week and to the spleen and distant lymph nodes in 2 to 4 weeks and that they may remain viable in the latter organs up to 6 months. Van Deinse(6)

obtained viable BCG from a suppurating lymph node in a child vaccinated by scarification 11 months earlier. The subject of BCG's viability and dispersibility in experimental animals and in man was recently reviewed by Fenner(7), and it appears that our finding viable BCG in the mesenteric lymph nodes 577 days after the intraperitoneal inoculation of 1 mg BCG in the guinea pig is the longest survival hitherto recorded without the organisms producing generalized tuberculosis(8). But these observations mostly dealt with sporadic findings and no long-term study is available on the viability and dispersion of BCG in animals inoculated with a fixed dosage of vaccine. The present investigation attempts to clarify this subject.

Experimental. In a recent work on uniform methods for continuous weekly production of BCG vaccine for use in man(9) we assessed 60 consecutive weekly lots of vaccine quantitatively for BCG's invasiveness and production of tissue hyperplasia in guinea pigs killed 3 and 6 months, respectively, after the subcutaneous inoculation of 10 mg BCG in the left hind leg. The inoculum abscess, regional and distant lymph nodes, the spleen, liver, and the lungs were removed and weighed to determine the degree of hypertrophy caused by a fixed amount of BCG. In the present study, the same procedure was followed, using 20 guinea pigs sacrificed at 3 months and another group of 20 killed 6 months after the subcutaneous inoculation in the left hind leg of 10 mg semi-dry weight of freshly prepared vaccine (Lots B92-B112: July 1950-April 1951). The average number of viable BCG (colonies) in these 20 weekly lots of vaccine was 43868000 ± 8603000/mg BCG. Careful *aseptic technic* was employed throughout. Separate instruments were used for the removal of individual organs. The entire lymph node, spleen, and inoculum abscess and approximately 2 g of the liver and lungs were finely partitioned with scissors and the macerated tissue placed individually in 1-ounce sputum jars containing approximately 20 g glass beads and supplied with a metal screw-top. The organs in the sputum jars were *homogenized* in a paint-shaking machine for 10 minutes. Ten ml of 4% NaOH were then added and the shaking repeated for 10 minutes. The homogenized material was centrifuged for 20 minutes at 3000 r.p.m. The supernatant was decanted and the sediment neutralized by 1.0, 0.1 and 0.01 normal HCl in the presence of 2 drops of phenol red indicator. After adding 200 units penicillin, the sediment was seeded in 0.2 ml amounts on 5 large tubes of Löwenstein-Jensen egg medium. The plugged tubes were left at 37°C in the horizontal position until excess liquid had evaporated (approximately 24 hours) when they were sealed with rubber stoppers. The tubes were left in the horizontal position for 1 week and in the vertical position for 7 weeks. The cultures were examined weekly for growth, the final reading being made after 8 weeks.

TABLE I. Comparative Wt of Organs in Normal and BCG Inoculated Guinea Pigs 3 and 6 Months, Respectively, after Inoculation. 20 animals in each group.

	3 months		6 months	
Organs	Normal, g	BCG inoculated, g	Normal, g	BCG inoculated, g
Body wt	674	654	826	796
BCG abscess	—	3.38	—	1.25
Lf. sup. inguinals	.07	.20*	.07	.23*
" deep "	$\leq$.01	.05*	$\leq$.01	.05*
" iliacs	.02	.11*	.03	.13*
Trach. bronchials	.08	.13*	.08	.15*
Cervicals	.06	.09	.07	.09
Axillaries	.05	.05	.05	.06
Spleen	.67	.79*	.69	.74
Liver	35.77	35.02	35.84	41.75
Lungs	4.73	5.64	5.04	6.52

* Significantly different from normal group at the $P \leq .01$ level.

Results. Table I presents data on weight of animals and 10 different organs removed from each of the BCG inoculated guinea pigs and also from a similar group of uninoculated animals. It is apparent that the weight of individual lymph nodes, especially those adjacent to the site of inoculation, was significantly greater in both groups of inoculated animals than in the controls. It is noteworthy that the weight of the spleen was significantly greater in the animals inoculated with BCG 3 months earlier than in the controls. These data confirm the relative but not absolute avirulence of BCG.

Table II presents data on the dispersion of

TABLE II. Survival of BCG in Organs of Guinea Pigs Inoculated Subcutaneously with 10 mg BCG 3 and 6 Months Earlier. 20 animals in each group.

Organs	Positive smears	Positive cultures	No. of colonies
	3 months animals		
BCG abscess	12/20	18/20	236 ± 167*
Lf. sup. inguinals	1/20	14/20	196 ± 152
" deep "	1/20	10/20	175 ± 196
" iliacs	0/20	9/20	171 ± 172
Trach. bronchials	0/20	13/20	141 ± 137
Cervicals	0/20	2/20	55 ± 50
Axillaries	0/20	3/20	70 ± 38
Spleen	1/20	7/20	316 ± 186
Liver	0/20	6/20	122 ± 74
Lungs	0/20	3/20	134 ± 107
	6 months animals		
BCG abscess	13/19	9/19	344 ± 130
Lf. sup. inguinals	1/20	6/20	203 ± 180
" deep "	0/20	5/20	264 ± 220
" iliacs	1/20	6/20	170 ± 176
Trach. bronchials	0/20	13/20	158 ± 191
Cervicals	0/20	5/20	166 ± 194
Axillaries	0/20	3/20	40 ± 33
Spleen	0/20	6/20	285 ± 209
Liver	0/20	3/20	40 ± 31
Lungs	0/20	3/20	211 ± 203

* Symbol ± stand. dev.

BCG from the inoculated area to regional and distant lymph nodes, to the spleen, liver, and lungs, as well as the rate of survival of BCG in these foci. It may be noted that the inoculum abscess yielded 60 and 68% positive smears of acid-fast organisms in animals killed 3 or 6 months after inoculation while positive smears were obtained irregularly from regional and distant organs. Positive cultures of BCG, on the other hand, were obtained from the inoculum abscess and regional lymph nodes twice as frequently in animals killed 3 months than 6 months after inoculation. However, the dispersion and survival of BCG in distant lymph nodes, the spleen, liver, and the lungs were approximately the same in both groups of inoculated animals. It is interesting that BCG survived more abundantly in the tracheo-bronchial lymph nodes in both groups of inoculated animals (65%) than in other distantly located lymph nodes since the hypertrophy of the tracheo-bronchial lymph nodes is also greater than that of other groups.

The average macroscopic growth of BCG colonies on the Löwenstein-Jensen egg medium was approximately the same in cultures of organs located regionally or distantly from the inoculum site. In spite of the generalized dispersion and long survival of BCG no macroscopic tuberculous lesions were abscessed except at the site of the inoculation and occasionally in the regional lymph nodes.

Summary. Twenty weekly lots of BCG inoculated subcutaneously in normal guinea pigs were widely dispersed and remained viable at site of inoculation and in distant lymph nodes, the spleen, liver and lungs up to 6 months without producing macroscopic tuberculous lesions except in the site of inoculation.

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Received April 6, 1952. P.S.E.B.M., 1952, v80.

Effect of An Arsenoso Compound in the Natural Infestation of White Mice With *Syphacia obvelata.* (19525)

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In an earlier paper(1) the marked anthelmintic effect of N-(p-arsenosobenzyl) glycine amide hydrochloride(2), designated Ro 2-1067, in the experimental trichinella infection of mice was described. A single oral administration of a well tolerated dose (500 mg/kg) of this arsenical protected the majority of mice against the fatal outcome of a massive infection with 2000 larvae of *T. spiralis* per mouse. A similarly striking effect was observed in the natural infestation of white mice with the pinworm *Syphacia obvelata.** Preliminary experiments had shown that the worm burden of naturally infected mice was completely eliminated by oral administration of Ro 2-1067. Following the procedure described by Wells(3) who used experimental infections with the mouse pinworm *Aspiculurus tetraptera* and Kam Fai Chan(4) who carried out similar experiments with Syphacia, repeated daily treatment for 5 to 6 successive days was employed in the earlier experiments. The success of this treatment schedule prompted a gradual reduction in frequency of drug administration until it was found that a single dose of the arsenical was sufficient to eliminate pinworms from the intestinal tract. The effect of graded single doses of Ro 2-1067 on the worm burden of the mice is described in this report.

Method. Adult albino mice of 22-26 g body weight were used in the experiments. The presence of Syphacia was ascertained by the demonstration of eggs in perianal smears using the Scotch tape technic. Groups of 20-36 animals received a single oral treatment by gavage with graded doses of the arsenical in aqueous solution and were then kept in cages in groups of 10-12 for a period of 6 days.

* We gratefully acknowledge the advice of Dr. Harry Most, Professor of Preventive Medicine, New York University, who drew our attention to the usefulness of this spontaneous infection for the study of anthelmintics.

TABLE I. Effect of Arsenical Ro 2-1067 in Infestation of Mice with *Syphacia obvelata.*

Ro 2-1067 dose, mg/kg	No. of mice	Avg No. of worms*	Cure rate†
	Exp. #1		
500	20	0	20/20
250	20	0	20/20
125	20	0	20/20
62.5	20	8.6	14/20
50	30	5.4	16/30
25	29§	24.1	6/29
	Exp. #2		
500	36	0	36/36
250	36	.05‡	35/36
125	36	1	31/36
62.5	36	1	27/36

* On 7th day after treatment.
† No. of parasite free mice/No. of treated mice.
‡ One mouse harbored 2 parasites.
§ " " died.

On the 7th day the animals were sacrificed and adult and larval forms of Syphacia present in cecum, colon and rectum counted.

Experimental. During the course of this investigation 418 normal not treated mice were examined which all had shown *Syphacia* eggs in the perianal smears. The autopsy of these animals revealed in 413 of them or 98.8% the presence of adult and larval forms of Syphacia in smaller or larger numbers. The average number was 44 parasites per mouse, but as many as 358 worms were found in one mouse.

The high degree of variation in the number of parasites which seems unavoidable in a natural infection made it advisable to base the evaluation of an anthelmintic effect on the complete disappearance of Syphacia from the intestinal tract. In Table I, in which the observations on the activity of Ro 2-1067 are given, the results of the average worm counts are supplemented by data as to how many treated animals were free of parasites.

It might be seen from the data in Table I that in the 2 experiments recorded a single dose of 500 mg/kg eliminated the parasites in all animals. Following smaller doses such as

125-250 mg/kg 86-100% of the animals were found free of Syphacia. If the dose was still lower (50-62.5 mg/kg) the number of parasite free animals decreased gradually and at the lowest dose level of 25 mg/kg almost all mice were found to harbor Syphacia. The number of worms recovered from the animals increased with decreasing doses of Ro 2-1067. It might be mentioned that in all treated mice as far as they were not completely free of *Syphacia* the number of larval forms was negligible (4 larvae in 79 mice) and they were, therefore, pooled with the adult forms.

Both the values of the worm counts and the cure rate appeared to be dependent on the dose of the arsenical used and indicate a definite dose-effect relation. The CD_{50} of Ro 2-1067 in the *Syphacia* infection of mice was estimated to be 48 mg/kg. Since the LD_{50} as determined for oral administration was 697 mg/kg the chemotherapeutic ratio LD_{50}/CD_{50} was 14.5.

In the experiments described so far the examination of the intestinal tract of mice was carried out 7 days after therapy. There is, however, evidence that the anthelmintic effect of a single administration of Ro 2-1067 is of longer duration. In one experiment a small group of 6 mice carrying *Syphacia* as shown by preliminary perianal smear was observed for 4 weeks after a single oral dose of Ro 2-1067, 500 mg/kg. The animals were kept without any special precautions in one of the commonly used suspended wire bottom cages. Only one of the mice contained 3 adult forms of *Syphacia* at the time of examination, the others were free of this parasite. Mice treated with Ro 2-1067 once and then kept in one cage together with infected mice were all found to contain *Syphacia* after 4 weeks. Protection against spontaneous infection with pinworms could, however, be achieved if the exposed animal received multiple treatments as in the following experiments:

A group of 14 mice with positive egg findings in the perianal smear were kept together in one cage. Nine of the mice received Ro 2-1067, 500 mg/kg by gavage twice a week, a total of 8 doses. Thirty days after the onset of the experiment and 3 days after the last treatment all treated and untreated mice were sacrificed and examined. In one of the treated mice one adult parasite was found, the other 8 were free of *Syphacia*. All 5 untreated animals harbored still parasites.

Discussion. It has been shown experimentally that a single treatment with the arsenoso compound Ro 2-1067 was able to eliminate *Syphacia obvelata* from infected mice. Ro 2-1067 was the first of our compounds to produce this marked effect which, as a rule, was not observed with most of the many other compounds tested. Known anthelmintics as far as they were active at all required repeated drug administration. The consistently high effect on *Syphacia* which can be achieved with a single administration of the arsenical suggests its usefulness for freeing mice of the parasite preparatory to their experimental infection with *Syphacia*.

Summary. N-(p-arsenosobenzyl)glycine amide hydrochloride possesses marked anthelmintic properties in mice infected with *Syphacia obvelata*. A single oral administration of 125-500 mg/kg eliminated the parasites in 85-100% of the animals. The CD_{50} was approximately 48 mg/kg.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

A Phagocytosis Inhibition Test in Infection Hypersensitivity.* (19526)

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(Introduced by W. J. Nungester.)

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A distinctive feature of infection hypersensitivity is cell response *in vitro,* as demonstrated by Holst(1), Rich and Lewis(2), Aronson(3), Moen and Swift(4), and others. In reporting their studies of sensitization in infection, most investigators have used tissue culture technics and have emphasized the importance of using cells from sensitized rather than normal animals. The addition of specific antigen to sensitized cells results in cessation of cell migration, along with necrotic changes. Favour and his co-workers(5) have suggested that the chief factor in these cell changes may be a component of serum from sensitized animals or man. These investigators have shown that serum from a hypersensitive individual plus the specific sensitizing antigen will cause lysis of either normal or "sensitized" leukocytes *in vitro*. Moreover, Favour(6) has reported that the serum factor responsible for cell changes is located in the globulin fraction, and that it is readily destroyed by heating at 56°C for 15 minutes.

The leukocytolysis reaction, while useful in studying infection allergy, requires an hour to complete and necessitates making two white cell counts. The work reported here is the result of efforts to devise a more rapid and more sensitive *in vitro* test for infection hypersensitivity. The possibility of inhibiting the phagocytic action of polymorphonuclear leukocytes with a serum factor and specific antigen was suggested by the fact that lysis of white blood cells undoubtedly is preceded by fundamental changes in structure of the cell membrane. Such changes might be expected to result in loss of specific cell functions before lysis would occur.

Materials and methods. Polymorphonuclear leukocytes were obtained by stimulating the production of an exudate in the peritoneal cavity of normal guinea pigs as described by Nungester and Ames(7). The cells were collected from the exudate, washed and diluted to a count of 25000/mm³. Their phagocytic action was tested in a system containing serum from animals sensitized to a beta-hemolytic streptococcus of Group B or to a Type I pneumococcus, and a filtrate of a 24 hour broth culture of the respective organism. The material to be phagocytized was a heat killed suspension of *Bacillus anthracis*. In making the tests 0.3 ml of sensitized serum was mixed with 0.1 ml of filtrate and 0.1 ml of the leukocyte suspension. The tubes were incubated with continuous agitation for 15 minutes at 37°C after which 0.1 ml of anthrax suspension was added with thorough mixing. A drop of this material was placed within a ring of petrolatum on a glass slide and overlayed with a coverslip. After an additional 15 minutes of incubation at 37°C, the slide was examined with the aid of darkfield illumination or a phase contrast microscope. Percentage phagocytosis was calculated according to the formula:

$$\frac{\text{No. of cells containing bacteria}}{\text{Total No. of cells counted}} \times 100$$

Normal serum, uninoculated broth, or 0.85% NaCl (in the combinations indicated in Tables I, II, III), were used as controls.

Results. Streptococcal filtrate alone had a slight toxic action but the combination of sensitized serum and specific filtrate caused a definite decrease in phagocytic action (Table I). Results of experiments using a Type I pneumococcus were essentially the same as those with the streptococcus (Table II). The specificity of the reaction is indicated by the failure to demonstrate inhibition of phagocytosis with sensitized serum and heterologous filtrate. Heating the sensitized serum at 56°C for 30 minutes destroyed its ability to prevent phagocytosis.

To demonstrate the association of phagocytosis inhibition with infection hypersensitivity, an attempt was made to block phago-

* This study was aided by a grant from the Lawrence J. Montgomery Bronchial Asthma Research Fund.

TABLE I. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Group B, β Hemolytic Streptococcus (Summary of 8 Exp.).

	Streptococcal filtrate, %	Uninoculated broth, %	.85% NaCl, %
Normal serum	76*	80	80
Sensitized serum	66	80	
" " (heated at 56°C)	79		

* % phagocytosis.

TABLE II. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Type I Pneumococcus (Summary of 3 Exp.).

Serum	Pneumococcal filtrate, %	Streptococcal filtrate, %	Uninoculated broth, %	.85% NaCl, %
Normal	86*		84	85
Sensitized	75	85		88

* % phagocytosis.

TABLE III. *In vitro* Inhibition of Phagocytosis in Hypersensitivity to Egg Albumin (Summary of 3 Exp.).

	Egg albumin 1:100, %	Egg albumin 1:1000, %	.85% NaCl, %
Normal serum	68*	73	85
Sensitized serum	75	84	83

* % phagocytosis.

cytic action with serum from guinea pigs sensitized to egg albumin. The results indicate that egg albumin, by itself, blocks phagocytosis (Table III). A similar observation has been reported by Ouweleen(8) in the phagocytosis of carbon particles but he could not show this blocking action of egg albumin when starch granules were used. In the present study, serum from sensitized animals neutralized the inhibitory action so that normal engulfment of the bacteria could take place.

Discussion. From the data presented it is seen that a factor present in the serum of guinea pigs sensitized to certain species of bacteria is capable of inhibiting the phagocytic action of normal guinea pig leukocytes in the presence of a filtrate of the specific organism. The materials used in this test are the same as those used in the leukocytolysis reaction, except that a suspension of bacilli was needed to demonstrate phagocytosis. This suggests that lysis results as an extension of the cellular changes which interfere with phagocytosis. The fact that the total white cell counts made at the beginning and at the end of some of our experiments showed a loss of 10-20% of the cells is further evidence for this view. Jung(9) and Hanks(10) in studies of quantitative phagocytosis, suggested that an appreciable drop in numbers of leukocytes would lead to an increased percentage phagocytosis, since the ratio of white cells to phagocytizable particles is altered. For this reason the 10% decrease (mean) which was actually observed in our experiments becomes even more significant.

All of the values presented in the tables are mean values for a series of experiments. To determine the possible significance of the 10% difference observed between sensitized serum plus filtrate and normal serum plus filtrate the standard error of the difference between the means (S. E.) was determined for the systems testing sensitization to streptococcus (Table I) and to pneumococcus (Table II). In the former instance the S. E. was 2.05 and in the latter was 1.89, indicating that the differences observed are statistically significant.

The specificity of phagocytosis inhibition is indicated by the results of tests using a heterologous filtrate (Table II). Failure to block phagocytic action with serum from guinea pigs sensitized to egg albumin in the presence of the specific antigen is in accord with findings using the leukocytolysis and tissue culture methods to study *in vitro* cell damage.

Summary. A phagocytosis inhibition test is described for infection hypersensitivity. It is similar to the leukocytolysis reaction and to studies of cell damage in tissue culture in that it is not readily demonstrated in hypersensitivity to egg albumin. The test is simple to perform and can be carried out very rapidly. These features, along with its specificity, make it a useful procedure for the *in vitro* evaluation of infection allergy.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Modification of Gomori Method for Alkaline and Acid Phosphatase Avoiding Artefact Staining of Nucleus.* (19527)

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The histochemical phosphatase methods originally outlined by Gomori(1,2) have been critically studied in this laboratory. Consistently reproducible results with Gomori's acid phosphatase method have been obtained during the past two years by adhering to certain details ascertained to be necessary in the procedure(3). By incubating the sections in the paraffin ribbons directly in substrate, the known harmful effect of exposure of the contained enzyme to various concentrations of alcohol incident to deparaffinization and rehydration was eliminated. Not only do the resultant preparations show greater and more constant preservation of phosphatase activity but also its localization is much sharper and appears in finer detail. In addition, the apparent phosphatase activity of the nucleus as seen in conventional preparations is largely if not entirely absent, as Ruyter and Neumann(4) have shown with the alkaline phosphatase technic. The discrepancy between the results obtained from cellular fragmentation experiments and from histochemical determinations is therefore resolved. As noted by several investigators (5,6), nuclei isolated by cellular fragmentation technics show chemically only a small fraction of the total phosphatase activity demonstrable. Histochemically this is confirmed by the Menten(7) or other similar methods which use the alcoholic end of the phosphate ester to produce a color reaction by formation of a diazonium salt. This is clearly seen in illustrations in the articles by Danielli (8) and Manheimer and Seligman(9) although no statement to this effect appears in their respective papers. Novikoff(10) however, has recently emphasized this point. The results obtained with the conventional Gomori method for alkaline or acid phosphatase(1,2) using deparaffinized tissue sections are in direct contrast to these data in that all nuclei in tissues showing phosphatase activity have also shown marked "phosphatase activity". This discrepancy is eliminated by the use of non-deparaffinized tissue sections in which it is found that the nuclei show very little activity. Since all 3 methods using entirely different approaches now agree it would seem that this localization is a true finding.

The validity of this unconventional method depends upon the assumption that the substrate reaches every part of the cell in which phosphatase activity may exist. This has been put to test by observing the penetration, or loss of substances of different molecular size into or from tissue sections within paraffin ribbons.

1) *Penetration of water.* As noted by Harrison and Bunting(11), ferrocyanide in paraffin sections of kidney is immediately removed by contact of the ribbon with water. Similarly, glycogen leaches out of paraffin sections mounted on a glass slide and immersed in distilled water for 2 hours, parallel-

* Supported by a grant from the National Cancer Institute, N. I. H., U.S.P.H.S.

† This work was begun while a fellow of the Jane Coffin Childs Fund.

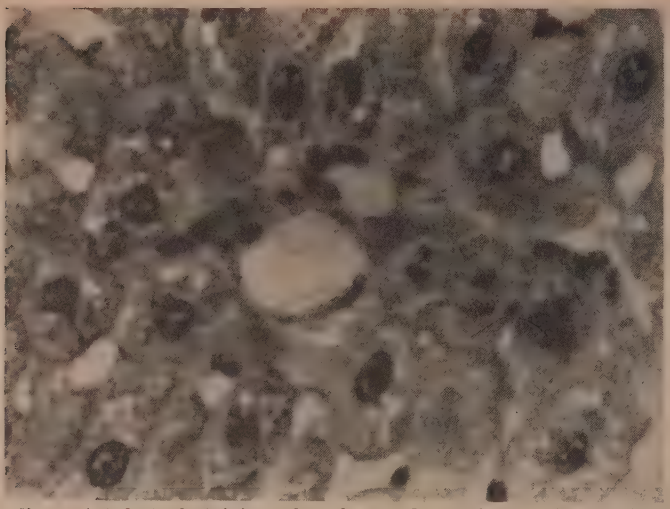

FIG. 1. Rat liver. Artefactual staining of nucleus and cytoplasm. Tissue boiled, embedded, sectioned. Sections soaked in Na_2HPO_4, then treated as routine alkaline phosphatase preparation. × 800.

ing the leaching seen in deparaffinized sections.

2) *Penetration of cobalt and or lead ions.* Paraffin sections of newborn mouse bone were mounted on glass slides and immersed directly in solutions of $CO(NO_3)_2$ at pH 9.4 and $Pb(NO_3)_2$ at pH 4.3 thus duplicating certain steps in the routine phosphatase technics. When subsequently treated with $(NH_4)_2S$ the sections showed precipitation of the metallic ions in the bone, the ions presumably having been bound to inorganic phosphate and carbonate there.

3) *Penetration of molecules* of acid and basic dyes. Excellent staining by acid and basic dyes of nuclear, cytoplasmic and noncellular structures can regularly be accomplished in non-deparaffinized sections by prolonging the exposure up to 18 hours. For the demonstration of this the following dyes and concentrations have been used: alcoholic eosin, 3%; aqueous eosin, 3%; aniline blue, 2%; Van Gieson (picric acid, acid fuchsin); light green, ¼%; paracarmine, 1-2%; basic fuchsin, ½%; toluidine blue, 0.05%; methylene blue, 2%; methyl green, 0.5%; Hematoxylin (Bullard's). It might be mentioned parenthetically that in these test preparations cytological distortion was appreciably less than in conventional deparaffinized sections. McFarland(12) advocated staining the paraffin ribbon as a short-cut in the preparation of histological material for student classes. Cowdry(13) described Mayer's stain for amyloid in which freshly cut paraffin sections are stained by immersion in 0.5% aqueous methyl or crystal violet for 5-10 minutes without removal of paraffin.

4) *Penetration of a section* under these conditions by a protein can be demonstrated by the rapid digestion by amylase of glycogen contained in liver cells in a non-deparaffinized section. Selective adsorption of the phosphatase enzymes by the nuclei has not been demonstrated by investigators. The cause of the apparent phosphatase activity of the nucleus in the sections prepared by the conventional methods is generally ascribed to the nuclear adsorption of calcium or lead phosphate resulting from phosphatase activity in the vicinity(10,14). A similar artefactual staining of the nucleus and to a lesser extent, of the cytoplasmic granules can be produced in the following manner: A mounted section in which the enzymes have been inactivated by boiling is soaked in a solution of Na_2HPO_4, then immersed half way in the alkaline phosphatase substrate buffer (without glycero-

phosphate). It is then treated successively in $CaCl_2$ in $CO(NO_3)_2$, and finally in $(NH_4)_2S$ as in the routine technic for alkaline phosphatase. In the region of the meniscus

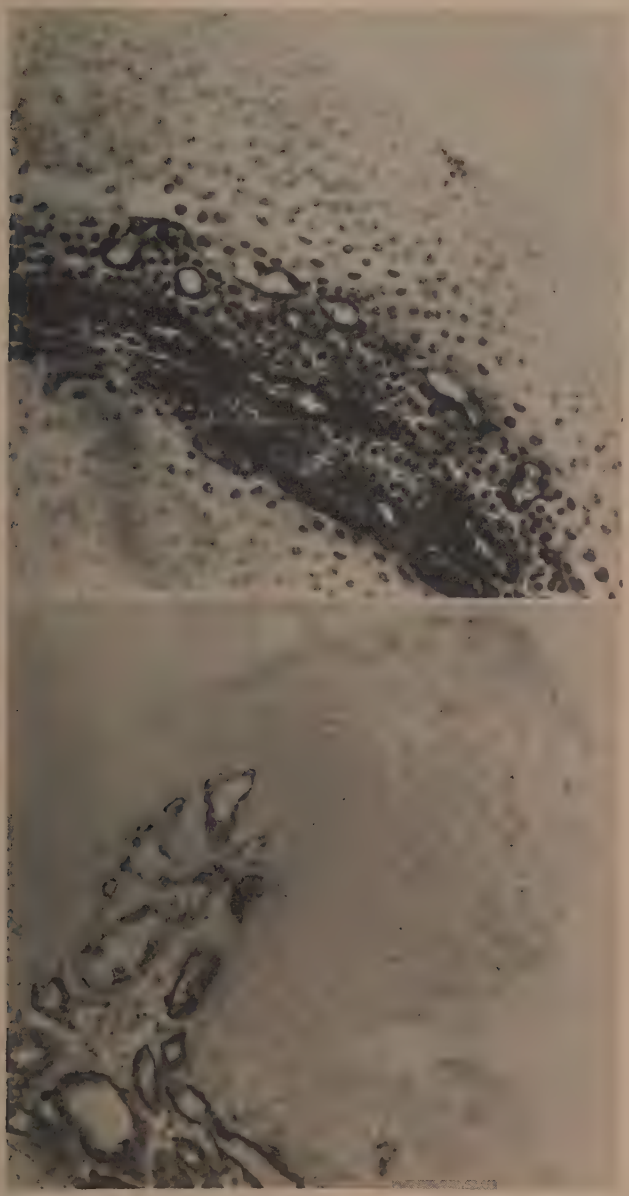

FIG. 2 (top). Human bladder papilloma fixed in 80% alcohol. Alkaline phosphatase preparation deparaffinized before incubation in substrate. Note gradient in nuclear staining from vicinity of capillaries toward periphery. Incubation time 6 hr. × 300.

FIG. 3 (bottom). Adjacent serial section to Fig. 2, incubated in substrate before removal of paraffin. Note lack of nuclear staining even adjacent to capillaries. Incubation time 6 hr. × 300.

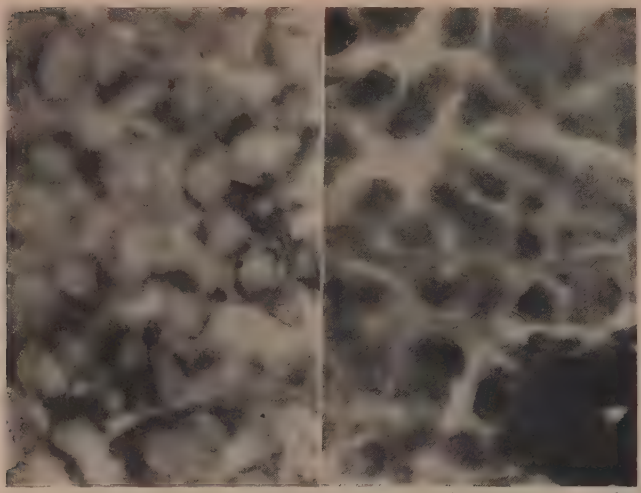

FIG. 4 (left). Rat spleen fixed in 80% alcohol. Germinal center, alkaline phosphatase preparation, incubated in substrate before removal of paraffin. Note lack of nuclear stain, presence of cytoplasmic activity. Incubation time 6 hr. × 1260.

FIG. 5 (right). Adjacent serial section to Fig. 4. Incubated in same substrate after removal of paraffin. Note the nuclear precipitate commonly interpreted as indicating nuclear phosphatase activity. Arteriole present in lower right. Incubation time 6 hr. × 1260.

which had been formed by the calcium-containing substrate buffer on the phosphate-soaked section the nuclei show a dense staining in a manner exactly similar to that seen in conventional alkaline phosphatase preparations (Fig. 1). The results are the same in this test whether the sections are or are not deparaffinized.

That much of the nuclear staining with the conventional Gomori technic is artefact is readily seen by comparing adjacent serial sections incubated in substrate, alternate sections having been deparaffinized. In the non-deparaffinized slides there is little if any diffusion within the section from points of high phosphatase activity such as capillary endothelium. In deparaffinized slides one can see a very definite gradient of staining density of nuclei from the points of high activity outwards. This is especially well demonstrated in tissues whose capillaries are widely separated such as in a papilloma of the urinary bladder (Fig. 2, 3). Martin and Jacoby(14) have demonstrated similar gradients in the nuclei of tissue having low phosphatase activity on which a tissue section having points of high activity had been superimposed. The non-deparaffinized sections may show at most a faint minutely granular precipitate associated with the chromatin network in the nucleus and the nucleolus of the fixed cell (Fig. 4, 6). This is in contrast to the dense, fairly uniform precipitate distributed throughout the entire nucleus seen routinely in the conventional Gomori technic (Fig. 5, 7). Histochemists have generally accepted this nuclear precipitate as denoting nuclear phosphatase activity. Further evidence that this is not the case is offered by careful inspection of conventional phosphatase preparations made from paraffin-embedded frozen-dried material deparaffinized before incubation. Here not all of the nuclei show the dense precipitate usually seen but many show staining only of the nuclear membrane, the nucleolus and very faint if any staining of the chromatin network.

The nuclei in frozen-dried preparations are often difficult to stain by the usual dyes just as in fresh frozen sections. This is presumably because the reactive surface groups have not been opened up and made available since there has been a minimum of denaturation of the proteins under these conditions as contrasted to the effects of the usual fixatives. The lightness of the staining of the nuclei in

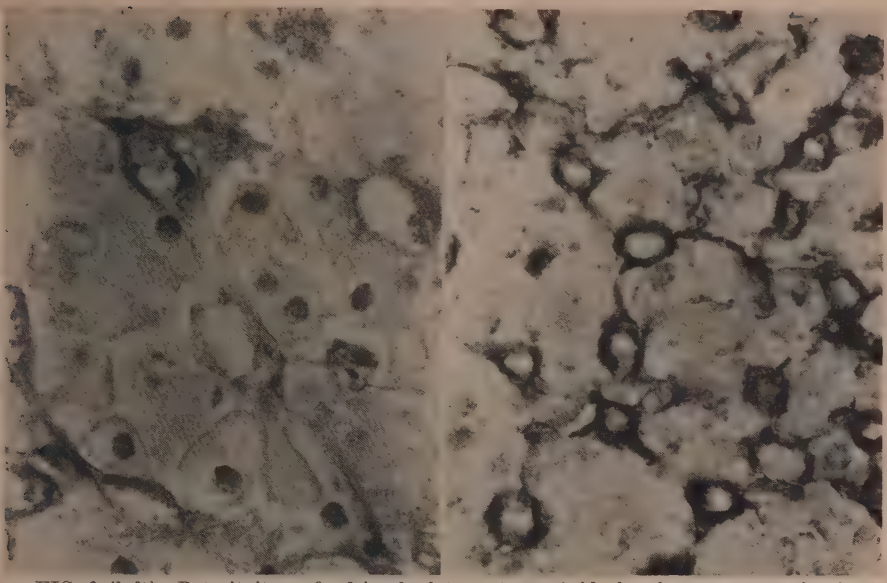

FIG. 6 (left). Rat pituitary, fixed in absolute acetone. Acid phosphatase preparation incubated after removal of paraffin. Note dense nuclear precipitate. Incubation time 6 hr. × 560.

FIG. 7 (right). Adjacent serial section to Fig. 6, incubated same length of time in same substrate before removal of paraffin. Note lack of nuclear staining and marked activity in capillaries. × 560.

phosphatase preparations in frozen-dried deparaffinized material contrasted with that after fixation with 80% alcohol would seem to be of similar origin, assuming that the staining here with Ca^{++} or $PO4^{---}$, or $Ca_3(PO4)_2$ is an acid-base phenomenon.

Summary and conclusion. 1. Staining sections before removal of the paraffin gives perfectly satisfactory, if not superior, results. Water and water-soluble material (dyes, substrate components, enzymes, etc.) readily penetrate the tissue structures exposed on the surface of the thin paraffin ribbon. The paraffin appears to act only as a filler which preserves the spatial relations of the various cytological elements during all staining and dehydration procedures. 2. Incubation of nondeparaffinized tissue sections in substrate gives a more accurate localization of phosphatase enzyme activity than the conventional method, and shows almost no activity demonstrable in the nuclei. It seems likely that the paraffin in the section prevents the lateral diffusion of the reaction products with their subsequent adsorption upon the nuclei. It is suggested this method be tried in other enzyme technics.

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Received April 17, 1952. P.S.E.B.M., 1952, v80.

Effect of Coagulase-Globulin on Residual Prothrombin Activity (Prothrombin Consumption) of Hemophilic Blood. (19528)

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It has been demonstrated(1) that coagulase-globulin produces a marked reduction of the coagulation time of hemophilic blood *in vitro* and *in vivo*, and that in hemophilic blood the titer of this factor is reduced. Since Brinkhous(2) has presented evidence that the lack of a plasma factor results in slow or incomplete prothrombin conversion, and Quick (3) has demonstrated that after hemophilic blood clots there is little reduction in serum prothrombin activity ("prothrombin-consumption"), the present study was carried out to investigate the changes in residual prothrombin activity after clotting of hemophilic blood was accelerated *in vitro* by the addition of coagulase-globulin.

Materials and methods. 1. *Normal whole blood* (N.W.B.), drawn from a normal adult subject using siliconized apparatus. 2. *Hemophilic whole blood* (Hem. B.), drawn from 2 hemophiliacs (No. 1 and No. 2) using siliconized apparatus. 3. *Saline*, 0.85%. 4. *Coagulase-globulin* (CG) prepared from fresh normal plasma as previously described(1) and redissolved in a volume of saline equal to the original volume of plasma from which it was prepared. 5. *Coagulation time* (C.T.): Blood from the siliconized syringe added to 3 glass tubes, 10 x 75 mm, for each determination, the coagulation time at 37°C being determined by the third tube after preliminary observation of the first two. 6. *Prothrombin activity*, according to the method of Quick (4, p. 142) by determining clotting time at 37°C of a mixture of 0.1 ml deprothrombinized rabbit plasma, 0.1 ml thromboplastin, 0.1 ml, 0.02 M $CaCl_2$, and 0.1 ml of serum. Serum obtained by centrifugation (1 min. at 3,000 r.p.m.) of each tube at 15-minute intervals after initial formation of solid clot.

Results. As shown in Table I, the pattern of serially determined residual prothrombin in normal serum(1) is significantly different from that in hemophilic serum(2,7). The addition of 0.2 ml of CG(4,9) produces a striking reduction in the coagulation time and also alters the pattern of residual prothrombin in the direction of normal. When the volume of CG is increased to 0.4 ml(5,10) the effect is even more pronounced. The addition of normal blood to hemophilic blood(6,11) has a similar but somewhat less striking corrective effect. Of interest are the observations(3,8) that the addition of saline to hemophilic blood not only reduces slightly the coagulation time but also significantly alters the pattern of residual prothrombin.

Discussion. The previous demonstration (1) that hemophilic blood is partially deficient in coagulase-globulin and that this plasma fraction is able to remedy the clotting defect *in vitro* and *in vivo* may be, at least in part, explained by the present study. Since residual serum prothrombin in hemophilic blood indicates little or no consumption, and since the addition of coagulase-globulin fraction alters this pattern in the direction of normal to the same degree as an equivalent amount of whole blood, then it seems justified to conclude that the factor supplied by coagulase-globulin as well as by whole blood is intimately associated with prothrombin conversion. Brinkhous(2) has concluded that the factor deficient in hemophilia is needed for platelet utilization. Quick(3) has reached essentially the same conclusion, and has suggested that the plasma factor (thromboplastinogen) reacts with the platelet factor (thromboplastinogenase) to form active thromboplastin. Although this reaction between platelets and coagulase-globulin is still under investigation, preliminary observations indicate that the plasma factor which reacts with platelets is probably coagulase-globulin. Although the purity of the coagulase-globulin has been previously defined(1) and leaves much to be desired, nevertheless, it has been shown to be free of calcium, prothrombin, thrombin, and fibrino-

TABLE I. Effect of Coagulase-Globulin on Residual Prothrombin Activity (Prothrombin Consumption) of Hemophilic Blood.

	No.	ml		ml	C.T., min	Min after formation of solid clot 15	30	45	60
						—Prothrombin activity, sec—			
1. N.W.B.		2			8	8	24	25	26
2. Hem. B.	1,	2			24	10	9	9	8
3.	1,	2	+ Saline,	.2	19	9	12	12.1	13.1
4.	1,	2	+ CG,	.2	7	9.8	16	16	16.5
5.	1,	1.8	+ CG,	.4	6	8.9	20	21.1	22
6.	1,	2	+ N.W.B.,	.2	14	10	13	13.5	14.5
7.	2,	2			135	12	11	11	11
8.	2,	2	+ Saline,	.2	110	10	12.9	15	15
9.	2,	2	+ CG,	.2	12	9.5	15	16	18
10.	2,	2	+ CG,	.4	9	10	17.5	18.5	20.1
11.	2,	2	+ N.W.B.,	.2	13	10	14.1	15.1	15.2

gen, and its deficiency in hemophilic blood suggests a specific deficiency of this plasma factor.

The observations that simple dilution of hemophilic blood with saline not only reduces its coagulation time in glass and in silicone tubes(1) but also alters the pattern of residual serum prothrombin activity (Table I, 3,8) have at the present time no ready explanation. Tocantins(5) has supposed that dilution disrupts an "accelerator-inhibitor complex" and that in hemophilia there is an excess of inhibitor, but the evidence for such an increase in inhibitor is not generally accepted. In any case, whether or not dilution disrupts normal or abnormal physico-chemical relationships, its effect is considerable, and it must be emphasized that great caution must be used in interpreting the results obtained from artificial systems, particularly in the *in vitro* assay of antihemophilic substances.

Summary. 1. The coagulase-globulin fraction of normal human plasma not only reduces the coagulation time of hemophilic blood but also alters the pattern of residual serum prothrombin activity in the direction of normal. 2. An equivalent amount of normal blood produces the same effect. 3. Simple dilution of hemophilic blood with saline by itself produces significant alterations in coagulation time and residual prothrombin.

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Received January 22, 1952. P.S.E.B.M., 1952, v80.

Experimental Hypersensitivity. Relationship of Dosage to Serological and Pathological Responses Following Injection of Heterologous Protein.* (19529)

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Although the histological response in the rabbit to injections of heterologous protein has been repeatedly described(1-5), the fre-

* This work was supported by a grant recommended by the Division of Research Grants and Fellowships, from the N. I. H., U. S. P. H. S.

quency of occurrence and the severity of the reaction has varied widely in these reports (6,7). An important variable has been the amount and type of protein used. As it is known that the frequency of serum sickness in humans is directly related to the amount of serum given(8), the same relationship might exist in the experimental model. Further, as the histological changes are evanescent and healing takes place promptly, the time of sacrifice would make uniform interpretation of the tissue response difficult. The purpose of this report is to determine how the amount of a uniform antigen would alter the serological and histological response in the rabbit.

Materials and methods. Animals. Male albino rabbits approximately 2 kg in weight were used in all experiments. *Serum protein.* Bovine serum gamma globulin (Lot Nos. C-1823B, C-188B and C-904) which had been prepared by the Armour Laboratories according to methods of Cohn and associates (9) was used as a 13.3% solution in 0.9% sodium chloride sterilized by Seitz filtration and used immediately. No preservative was added. *Sheep cells.* The sheep blood was drawn aseptically and preserved in modified Alsever's(10) solution. The cells were not used beyond the age of 6 weeks. When used, the cells were washed 3 times in the phosphate buffer, centrifuging at 2000 rpm for 10 minutes. Approximately 2% suspension of cells was made by adjusting the concentration so that 0.4 cc of the suspension when hemolysed with distilled water gave a reading of 700 on the Coleman Junior spectrophotometer at a wave length of 545 μ. This corresponds to a cell suspension of approximately 500000 cells per cu mm. *Diluent.* Phosphate buffer as described by Kent *et al.*(10) was used throughout. *Hemolysin.* Commercial hemolysin (Lederle, Lot Nos. 121A and 122A) prepared by the usual procedure was used. The optimal amount for sensitization of sheep cells was determined by titration as described by Kent(11). *Complement.* Blood was drawn from the marginal ear vein of the rabbit. This was spun in a refrigerated centrifuge at 0°C and the serum was removed and stored in paraffin-sealed tubes at minus 23°C until used. Complement titrations of all the

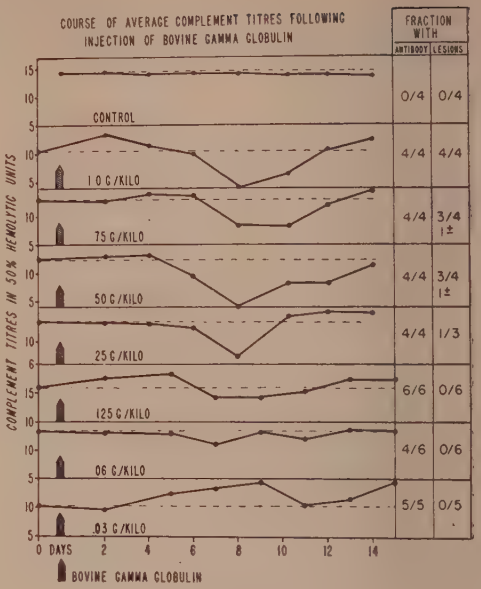

FIG. 1.

serum samples for a single animal were run at the same time. Titrations of complement modified for the Coleman Junior spectrophotometer were made as previously described(12). *Antibody.* Quantitative precipitin was obtained using the procedure of Heidelberger and Kabat(13,14) and the amount was determined by the ultraviolet absorption spectroscopy method of Gitlin(15). The extinction coefficients determined and used were 14.4 for rabbit antibody and 13.4 for the bovine gamma globulin antigen. When serum was sufficient the determinations were made in duplicate on 0.5 and .75 ml of serum; with insufficient serum for a satisfactory run, the ring test(1) was done. *Histological studies.* As in previous experiments, complete autopsies were made on the animals, but only the presence or absence of renal lesions are here recorded since it has been our experience that these histological changes are the most consistent of the several lesions attributable to experimental hypersensitivity.

Observations. Seven series of rabbits were given graded intravenous injections of bovine gamma globulin. The amounts of globulin were 1.0, .750, .500, .250, .125, .060, .030 g per kilo body weight. The complement titers

TABLE I. Effect of Graded Injections of Bovine Gamma Globulin on Time and Amount of Antibody Production.

Amount, g/kilo	Animal	Antibody in μg AbN/ml — Days after inj. 6	8	10	12	14	Renal lesion
1	28			++	18	18	Pos.
	30			19	++	++	"
	27				±	13	"
	29				12	18	"
.75	15			+	16	26	"
	21			12	14	++	Equivocal
	11				+	++	Pos.
	7				+	++	"
.50	25		±	25	20	++	"
	26			15	23	19	"
	23			11	++	18	Equivocal
	24				±	14	Pos.
.25	10		++	+++	+++	38	Neg.
	18		±	++	18	30	"
	8			+	14	18	Pos.
	19			8	11	27	Lost
.125	80	13	15	++	++	13	Neg.
	84		±	6	11	+	"
	85		8	15	14	++	"
	81			±	±	±	"
	82			±	±	+	"
	83				±	+	"
.06	88	11	—	++	—		"
	89	27	24	24	23	++	"
	86		11	12	15	+	"
	87			±	±	+	"
	90						"
	91						"
.03	93	15	30	26	18	12	"
	94		+++	+++	25	17	"
	97		±	±	±	++	"
	99		++	++	++	++	"
	95			++	++	+	"

and antigen or antibody were determined approximately every 2 days until sacrifice on the fourteenth day. From Fig. 1, it can be seen that the averages of the blood complement titers show a definite transient depression on about the eighth to tenth days in those groups given 1.0, .750, .500, and .250 g per kilo body weight. The averages tend to obscure the abrupt and striking fall in the serum complement in individual rabbits but even with the averages plotted, the fall is apparent only in those receiving the large amounts. When amounts given were .125, .060, and .030 g per kilo, the depression in complement is not regularly demonstrable in individual titrations and does not appear upon averaging the values.

Antibody formation occurred regularly in all animals except 2 in which neither antigen nor antibody were demonstrable after the ninth day following .060 g per kilo of the antigen. Table I illustrates the time and amount of circulating antibody found. In general, the smaller the amount of antigen injected, the earlier was the appearance of circulating antibody. Although there is great individual variation, the antibody nitrogen values showed no clear difference which could be related to the amount of antibody found and the presence or absence of histological changes.

The histological changes seen are recorded in Fig. 1. Renal lesions were frequent in the animals receiving larger amounts of antigen but were less common as the amount of antigen was reduced. The lesions could be seen nearly regularly in animals given 1.0, .750, and .500 g per kilo, in one animal given .250 g, and

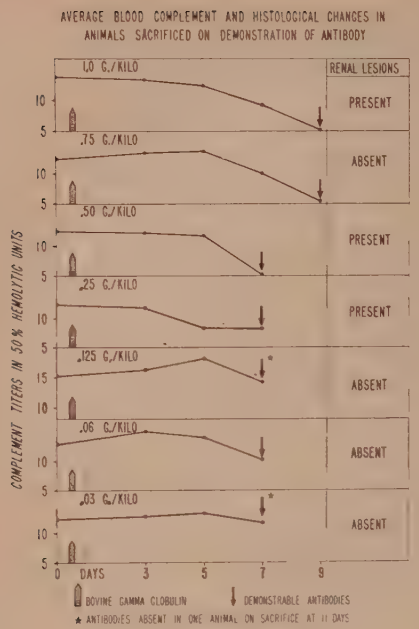

FIG. 2.

none in the animals given less than this amount. As the lesions are evanescent and because in those receiving a smaller injection antibody formation could be detected early, two animals in each group were sacrificed on the first or second day after appearance of demonstrable antibody in the circulating blood. The results of this study are seen in Fig. 2. Again renal lesions were seen in the more heavily injected group and no change attributable to hypersensitivity could be seen in those given less than .250 g per kilo body weight.

Antibody formation was always demonstrable in those animals which developed lesions and all such animals showed a depression of the circulating complement just at the time of the appearance of circulating antibody. The precipitin could be demonstrated when the complement was at its lowest level or immediately thereafter, as can be seen most clearly in Fig. 2. However, although the drop in complement was always associated with appearance of precipitins, its depression also occurred sporadically in those rabbits in which renal lesions were not demonstrable.

Discussion. Reports of the regular production of histological changes following the injection of heterologous protein have all involved the use of large quantities of antigen. More and Waugh(4) used 1.0 g per kilo of bovine gamma globulin. Hawn and Janeway (1) gave the same amount of bovine gamma globulin and bovine albumin. Rich and Gregory(3) used 10 ml per kilo of horse serum. Ehrich(2) *et al.* gave both 10 ml and 20 ml horse serum per kilo and observed that the larger dose was more effective. Further, it has also been shown that the specific protein used alters the frequency and location of histological changes(1).

From the work presented herein, it would appear that the amount of antigen injected directly influences the incidence of the renal lesions seen, and these changes are not related to the level of circulating antibody produced. This supports the observation of Rich and his coworkers(16) who found no correlation between the antibody titers and the appearance of lesions, but in contrast to Cohen *et al.* (17) who felt that the lesions were a function of antibody rather than antigen levels.

The depression of circulating blood complement occurring at the period when histological changes are most acute may represent its utilization in the interaction of antigen and antibody(12). This depression was most clearly evident in the animals receiving the larger amounts of antigen. Moderate declines were occasionally observed in some of the animals receiving the smaller amounts of antigen and these declines were regularly followed by the appearance of precipitins but without histological alterations.

From the evidence presented, the speculation is warranted that only when large amounts of antigen are used is the interaction of antigen and antibody prolonged and of such magnitude that the interaction is severe enough to produce histological changes and depletion of complement is detectable. The level of the circulating antibody may be merely an index of the amount of antibody in excess of that needed for neutralization of the antigen circulating and in the tissue, and may bear no direct relation to the histo-

logical changes seen.

Summary. Forty-seven rabbits were given graded injections of bovine gamma globulin intravenously. These were bled frequently for antibody and complement studies and then sacrificed for histological examination. When amounts used were from .250 g to 1 g per kilo body weight, glomerular lesions attributable to hypersensitivity were seen in most animals, and regular depression of complement followed by the appearance of circulating antibodies was demonstrable. In smaller doses, no histological changes occurred and the serological alterations were inconstant.

The authors wish to express their appreciation to Dr. Charles A. Janeway and Dr. John F. Enders of the Children's Medical Center, Boston, Mass., for their help and criticism in the preparation of this manuscript.

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Received March 4, 1952. P.S.E.B.M., 1952, v80.

Induction of Necrosis in Mouse Mammary Carcinoma by Cortisone.* (19530)

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The use of 11 dehydro 17 oxycorticosterone (Cortisone) in attempts to produce regression of neoplasms is not new. Heilman and Kendall(1) reported temporary regression of a mesenchymal tumor in mice after administration of this agent in doses smaller than those used in the present study. They did not report on the nature of the damage to the neoplasms. Vrat(2) has reported that cortisone has no effect upon mouse mammary carcinoma. The dose levels he used were smaller than those employed in our studies. There has been extensive clinical use of cortisone in the treatment of various types of neoplasms in man. The results have been disappointing as to long-term effects in spite of evidence of temporary remissions.

There is a large literature(3) on the use of bacterial toxins, colloidal substances of numerous sorts and other toxic agents in producing intra-tumoral hemorrhage and necrosis in man and other animals. In general it can be said that numerous such agents induce injury to neoplasms, greater in extent and degree than that inflicted on the host, but that no agent is known which regularly causes total

* Aided by grants from the Minnesota Division American Cancer Society, the U. S. Public Health Service and the Graduate School Medical Research Fund.

TABLE I. Effects of Cortisone at Various Dose Levels on Mice with Spontaneous Mammary Carcinoma.

No. of mice	Cortisone, mg/mouse	Days of treatment	Tumor necrosis No.	Tumor necrosis %	Generalized reactions No.	Generalized reactions %
3	.50	10	0	0	0	0
12	1	4	2	17	1	8
12	2.50	4	3	25	1	8
41	5	4	14	34	12	29
14	10	4	8	57	6	43

TABLE II. Effects of Cortisone and of Terramycin upon ZBC Mice Bearing Transplanted Z-9168 Mammary Carcinoma.

Tumor transplant generation	No. of mice	Cortisone, mg/mouse /day	Days of treatment	Tumor necrosis No.	Tumor necrosis %	Generalized reactions No.	Generalized reactions %
2nd	17	5	4	5	29	5	29
3rd	12	5	4	4	33	2	17
4th	8	5	4[T]	6	75	0	0
4th[E]	13	5	4[T]	13	100	0	0
5th	17	5	4[P]	9	53	3	18

[T] Terramycin in drinking water .1%, 6 days beginning day prior to cortisone treatment.
[P] Penicillin inj. 1000 units/day, 6 days beginning day prior to cortisone treatment.
[E] Treated also with tumor extract (see text).

tumor destruction in non-fatal doses. In a few instances, both in man and in other animals, it appears that complete regression has occurred with host survival. A safe dose level for regular results of this type has not, however, been found for any substance or physical agent. Therefore further search for agents or combinations of agents capable of producing such results is not only warranted but urgently needed.

Methods and materials. Both transplanted and spontaneous mouse mammary tumors have been employed in these studies because in some respects the transplanted tumor allows more simple experimentation and interpretation. For work with transplanted tumors female ZBC animals were employed, using a spontaneous tumor derived from a Z animal. This tumor will be identified as Z-9168. Transplantations were made to the right axilla of ZBC mice by the cell suspension technic. The animals were employed for study when the tumors reached approximately 1 cm diameter. In other studies spontaneous mammary tumors from the A, Z, D, and ZBC stocks were employed. In general the tumors were more than 1 cm in diameter at the time of use. After experimentation mice were sacrificed at various intervals of time and subjected to gross and microscopic examination of their mammary tumors and their viscera. Other details of experimental procedure will be indicated in connection with the results.

Results and discussion. Table I presents the observations upon mice bearing spontaneous mammary tumors treated with various dose levels of cortisone. With increasing doses there is an increase in the proportion of mice showing gross tumor lesions. The character of the tumor lesion as seen on microscopic examination was a combination of hemorrhage and necrosis. The necrotized areas showed disorganization of tumor cell arrangements, detachment of cells, infiltration with leucocytes and nuclear pyknosis. The extent of the lesions varied from 25 to 90% of the tumor mass. Complete destruction was not observed.

In addition to the tumor injury the mice showed destructive lesions in lungs, liver and kidneys. Such effects, which are lethal unless treated as noted below, have been reported before(4). The frequency of these findings, listed as generalized reactions, also increased with cortisone dose level.

In Table II are shown the summarized results of experiments upon ZBC mice with transplanted tumors. In 25 such mice one case of spontaneous tumor necrosis was observed, therefore it would appear that any incidence higher than 4% in this tumor can be

ascribed to treatment. It will be noted that the proportion of animals showing gross tumor lesions ranges from 29 to 75%, excluding the case of mice which had in addition an intravenous injection of a Seitz filtrate of a 0.9% NaCl extract of homologous tumor tissue. Likewise as to generalized reactions the frequency was of the same order of magnitude as was found with spontaneous tumors at the same cortisone dose level, except where supplementary treatment was given.

When terramycin, 0.1% in the drinking water was given the incidence of generalized reactions fell to zero. Penicillin in the dose employed did not show such an effect. Two points are of interest in this connection. First, it is apparent that terramycin did not reduce the frequency of tumor necrosis following cortisone. Second, it is apparent that terramycin reduced the frequency of generalized reactions and allows long survival.

The fact that survival can be expected following tumor necrotizing doses of cortisone when accompanied by terramycin suggests that by combining with such treatment another agent differentially more damaging to tumor than to host tissues, more complete tumor destruction might be accomplished. Studies using radiation, chemical agents and antigenic materials to such an end are already in progress.

Conclusions. In mice bearing spontaneous and transplanted mammary carcinoma it is possible by treatment with cortisone to induce a high frequency of massive necrosis with or without hemorrhage in the tumors. Cortisone administration in necrotizing doses is also followed by inflammatory lesions in lungs, liver and kidneys, which are usually fatal. Terramycin treatment before and during cortisone administration resulted in an abolition of the above-mentioned generalized reactions. It has not been possible with cortisone alone or with terramycin to obtain complete tumor destruction. It is pointed out that cortisone necrotizing treatment in conjunction with some other agent having differential damaging effects upon tumor and host respectively, may yield better results.

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Received March 26, 1952. P.S.E.B.M., 1952, v80.

Differentiation of Growth Hormone from the Pituitary Factor Which Produces Diabetes. (19531)

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The diabetogenic effect of pituitary extracts, observed by Houssay(1), was attributed by Evans to the growth hormone(2). It is now generally accepted, despite earlier evidence to the contrary(3), that growth hormone is the factor in pituitary extracts which is responsible for the production of diabetes(4-7). Extensive experimentation has yielded clear evidence that the pituitary contains a potent diabetogenic agent, or combination of agents, which rapidly produces severe insulin-resistant diabetes when administered to susceptible animals, and which produces permanent diabetes with pancreatic damage after prolonged administration(2,8). The diabetogenic effect may be obtained in the

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adrenalectomized dog(9) and is, in the intact animal, a far more potent effect than is induced by corticotropin, which may cause transient hyperglycemia. The acceptance of growth hormone as the pituitary diabetogenic factor stems largely from the belief that the purified growth hormone preparations which produce the effect, including those which are crystalline and electrophoretically homogenous(10,11), are indeed chemically and biologically pure. Some further evidence, based on the concomitant destruction of growth-promoting and diabetogenic activity, has suggested that the production of diabetes is an inherent property of the growth hormone(12).

It has now been found that the growth-promoting and diabetogenic activities of pituitary extracts may be differentiated, and that growth hormone, prepared as recently described(13), is largely freed of the diabetogenic factor.

Experimental. Preparation of growth hormone: As in the method of Astwood, Raben, and Payne for the purification of corticotropin (14), acetone-dried pig anterior pituitary powder† was extracted at 70°C with glacial acetic acid, thyrotropin, luteotropin, and gonadotropins were removed by precipitation with a half-volume of acetone in the presence of salt, and "crude corticotropin" was precipitated with one volume of ether(15). Two per cent of the solids containing 90% of the corticotropin was removed by stirring a 2.5% solution of the ether precipitate in 0.1 N acetic acid with an 8% weight of oxycellulose (12% carboxyl) for 24 hours(16). Retreatment with oxycellulose using a 20% weight of the adsorbent removed another 1% of the solids and much of the residual corticotropin. The unadsorbed fraction, largely freed of other pituitary hormones, contained the growth hormone, which was concentrated by a modification of Wilhelmi's procedure(11). To the solution of unadsorbed material was added, with vigorous continuous stirring, strong potassium hydroxide calculated to make the solution 0.3 N with respect to potassium. Glacial acetic acid was then immediately added until a permanent cloud formed. The pH at this point was about 10. Additional glacial acetic acid was slowly added until the pH was brought down to 8.5. Stirring was continued for one-half hour and the precipitate then removed by centrifugation. The procedure to this point was carried out at room temperature but the subsequent steps were performed in the cold. To the supernatant of the pH 8.5 precipitate, while being stirred in an ice-water bath, was added an equal volume of 95% ethyl alcohol. The addition of alcohol was made over a period of 15 to 30 minutes, and stirring was continued for an additional 30 to 60 minutes. The preparation was then allowed to stand overnight in a cold room at 4-5°C, and the precipitate was then collected on a sintered glass filter, washed with 95% alcohol and with acetone, and dried in a vacuum. This precipitate contained the growth hormone and represented about 12 to 15% of the total material in "crude corticotropin." The preparation was readily dissolved by acidification with hydrochloric acid to about pH 3.5; no loss of potency was noted when a solution was kept at this pH. The growth hormone preparation thus obtained was found by assay in the hypophysectomized rat to be about equal in growth-promoting activity to the preparations of Li and of Wilhelmi, and to the Armour growth hormone preparation (Lot 285-110)‡ with which it was compared for diabetogenic activity.

Estimation of diabetogenic activity. When 3 adult dogs injected with this growth hormone preparation in doses up to 10 mg/kg/day failed to develop glycosuria, the preparation was compared for diabetogenic activity with the Armour growth hormone preparation. A male dog weighing 8 kg (Fig. 1) after receiving single daily injections of 50 mg of the present growth hormone preparation for 6 days, and 100 mg for 2 days without glycosuria, was given the Armour preparation in a dose of 50 mg/day. Glycosuria appeared after the third injection and continued until the injections were stopped. Retreatment with our preparation in doses of 100 mg/day for

† We are grateful to Dr. David Klein of the Wilson Laboratories for supplies of pituitary powder.

‡ Kindly provided by Dr. E. E. Hays of the Armour Co.

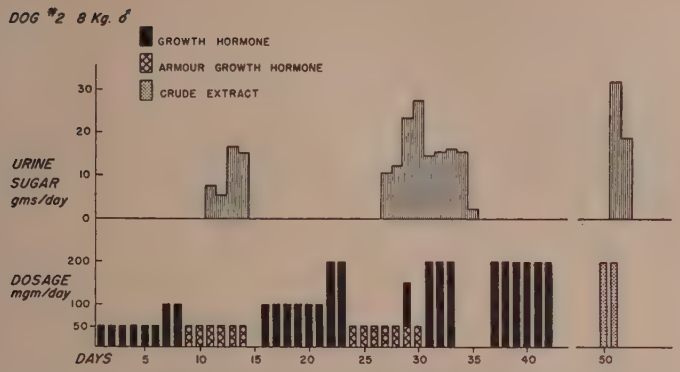

FIG. 1. Comparison of growth hormone prepared as described in the text, an Armour growth hormone preparation, and a crude extract in the production of glycosuria.

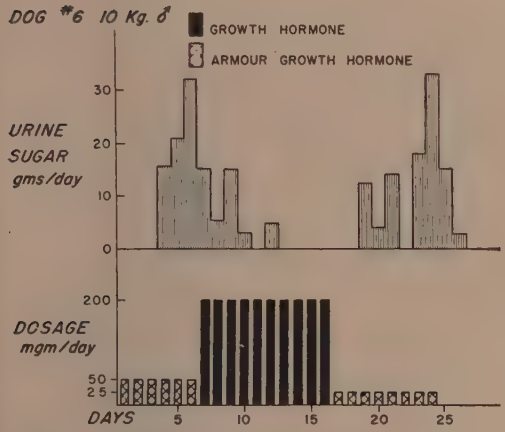

FIG. 2. Comparison of the growth hormone preparation and an Armour growth hormone preparation in the production of glycosuria.

6 days, and 200 mg/day for 2 days, again produced no glycosuria. A second course of the Armour material in a dose of 50 mg/day produced glycosuria beginning with the fourth injection. Glycosuria did not reappear during a third period of treatment with 200 mg/day of the present preparation, but did occur with the subsequent administration of the crude glacial acetic acid extract.

In another experiment carried out on a male dog weighing 10 kg (Fig. 2), the glycosuria which developed with the fourth injection of 50 mg of Armour growth hormone disappeared during 10 days of administration of the present preparation in a dose of 200 mg/day and recurred with Armour growth hormone in a dose of 25 mg/day.

Discussion. The difficulties involved in determining purity of pituitary hormones have been emphasized by recent experience with the purification of corticotropin(14). Isolation of the hormone was thought to have been achieved with preparations which were only 1% as potent as those subsequently produced. It now appears that diabetogenic material is present as a contaminant in growth hormone preparations which are crystalline and which behave as homogeneous substances when studied by solubility, diffusion, ultracentrifugation, and electrophoresis(10,11,17). The growth hormone prepared by the present method is probably not entirely freed of the diabetogenic principle, for large doses, while not producing glycosuria, did seem to delay the disappearance of established glycosuria (Fig. 1 and 2). The separation of the growth hormone from the factor which produces diabetes is nearly complete, however, and indicates the separate identity of these substances. The differentiation of the two activities serves to resolve the inconsistency in the concept that the metabolic processes which lead to growth also lead to diabetes.

Summary. Growth hormone was prepared from a glacial acetic acid extract of anterior pituitary powder. The product was equal in growth-promoting activity to other purified preparations but did not produce diabetes when administered in relatively large doses to

dogs, indicating that the diabetogenic factor of the pituitary is distinct from the growth hormone.

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Received April 4, 1952. P.S.E.B.M., 1952, v80.

Glucose Absorption in Highly Inbred Strains of Mice.* (19532)

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Studies on glucose absorption in the rat, beginning with the work of Cori(1), have raised a number of questions chief among which is the relation of absorption rate to the concentration of the administered solution. Cori(1) and others have claimed that absorption is essentially independent of concentration, while MacKay and Bergman(2), Fenton (3), and others found absorption to increase with increasing concentration of the administered solution. Two different colonies of rats were used by Fenton(3) with differing results. While both colonies showed dependence of absorption rate on concentration, this was much less pronounced in one of the colonies studied. It seemed that this colony difference might account for the divergent results which have appeared in the literature and might best be investigated in mice of highly inbred strains and of known genetic background.

* Supported by grants from the Fluid Research Fund, Yale University School of Medicine, the Anna Fuller Fund, and the American Cancer Society.

The demonstration that glucose absorption is accomplished by phosphorylation has led to speculation on the role of the widely distributed alkaline phosphatase in the absorption process(4). It was decided to investigate changes during glucose absorption in the concentration of phosphatase (using chemical methods) and in distribution (using cytochemical technics).

Methods. Female mice of the C57 and A strains, approximately 6-8 months old were fasted for 24 hours. Both strains were derived from the colony of Dr. L. C. Strong, Yale University, and were maintained by brother-sister matings. Following the fasting period 0.25 ml of glucose solution (25, 50 or 75% concentration) was administered by stomach tube. Exactly one hour later the animals were sacrificed and the contents of the alimentary tract (stomach, small and large intestines) washed into a volumetric flask. The glucose content of these washings was determined by the method of Somogyi(5). A group of 23 mice was fasted and then sacrificed without

TABLE I. Milligrams Glucose Absorbed per Hour.

Strain	No. of animals	Glucose conc., %	Glucose administered, mg	Glucose absorbed, mg
C_{57}	19	25	62	57
A	6	25	62	56
C_{57}	25	50	121	79
A	9	50	121	80
C_{57}	19	75	184	109*
A	25	75	184	97*

* $p = .05$.

TABLE II. Phosphatase Activity of Intestinal Homogenates.

	Solution administered	No. of animals	Phosphatase activity*
A.	0	8	28.7
	.5 ml 25% glucose	5	27.6
B.	0	10	21.4
	.5 ml 25% glucose	10	18.3

* Expressed as mg phenolphthalein liberated by homogenate from one entire small intestine.

glucose injection. The reducing power of the gastrointestinal washings was determined and used to correct the values for unabsorbed glucose found for the injected animals. The exact glucose content of the administered solution was determined each time an experiment was carried out. Phosphatase activity of the small intestine was determined by the method of Huggins and Talalay(6) using a homogenate prepared with distilled water. Control mice were fasted 25 hours before being sacrificed. The experimental animals, fasted 24 hours, received 0.5 ml of 25% glucose solution by stomach tube and were sacrificed one hour later. In pilot experiments (Group A, Table II) the entire small intestine with its contents was homogenized. In later work (Group B, Table II) the small intestine was washed out before being homogenized. A cytochemical study of alkaline phosphatase activity in the duodenum was carried out by the method of Gomori(7).

Results. The absorption data are summarized in Table I. The only significant strain difference in absorption rate was noted with the 75% glucose solution. It should be noted that increasing the concentration of the administered solution increased the absorption rate. Animals which had been raised on synthetic diets showed absorption rates identical with those raised on stock diet and are therefore not included in the table. Data obtained with mice of the C_3H strain are similarly omitted because they were virtually identical with those from A strain mice. Residual reducing materials present in the alimentary tracts of fasting mice were equivalent to 3.9 mg of glucose.

Alkaline phosphatase activity of the small intestine determined chemically was only slightly less in animals fasted and then fed 0.5 ml of 25% glucose than in animals sacrificed after fasting (Table II). Histochemically some slight differences were detectable. Fasted animals showed intense phosphatase reactions at the cuticular borders of the mucosal lining cells with considerable reaction in the Golgi zone. The rest of the cytoplasm gave a faint reaction which was more intense in the apical than in the basal portion of the cells. Administration of 0.5 ml of 0.9% NaCl solution by stomach tube did not change this picture markedly. Similar feeding of 25% glucose usually led to a diminution of phosphatase reaction in the Golgi zone and slight intensification of the cytoplasmic reaction in the apical portion of the cell. Administration of 60% glucose produced the same shift of cytochemically determined phosphatase but in addition caused marked desquamation and shrinkage of the surface epithelium. Administration of solutions of arabinose, glycine or corn oil produced little change from the picture seen in fasted, untreated animals. Mice fed corn oil showed, however, clearly visible phosphatase reaction in the lacteals.

Discussion. Since it has been shown in an earlier publication(3) that the intestinal absorption of glucose depends very largely upon the rate of gastric emptying, the strain difference in absorption of 75% glucose solutions (Table I) might be ascribed to the slower gastric emptying of such solutions in the A strain mice. This in turn is likely to be a reflection of the degree of inhibition which concentrated glucose solutions are known to induce (enterogastrone, nerve reflexes). Mice of the A strain might then be thought of as being somewhat more sensitive to such inhibition than mice of the C57 strain. If such strain differences are at all common, it is

conceivable that the animals used by Cori might have been so sensitive to inhibition that the progressive reduction in rate of emptying of the stomach with increasing concentration of solution fed might abolish the usual concomitant increase in amount absorbed.

Chemical determinations of phosphatase activity of intestinal homogenates showed that a considerable amount of the enzyme is present in the fluid contents of the small intestine (compare groups A and B, Table II). Oral administration of 25% glucose probably decreases the amount of enzyme in the tissues and increases the amount in the intestinal fluid. The effect is small. The cytochemical study, in agreement with the chemical findings, showed some translocation of the enzyme but no dramatic changes in concentration.

Conclusions. 1. Glucose absorption in the mouse has been studied. Increasing the concentration of administered solution results in an increase in the amount of glucose absorbed. 2. Glucose solutions of 75% concentration were absorbed somewhat more readily by mice of the C57 strain than by those of the A strain ($p = 0.05$). 3. Administration of 25% glucose solutions leads to some diminution of the phosphatase activity of the small intestine determined both chemically and cytochemically.

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Received April 4, 1952. P.S.E.B.M., 1952, v80.

Prolongation of Effects of Adrenal Cortical Secretion by Ascorbic Acid: Proposed Mechanism of Action.* (19533)

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(Introduced by C. E. Leese.)

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Systematic studies in this laboratory indicated an intimate relationship between ascorbic acid and the activity of the pituitary-adrenal axis. The alarm reaction (eosinopenia, lymphopenia, and adrenal cholesterol depletion) characteristic of acute exposure to stressors (*e.g.* epinephrin, trauma) was prevented by ascorbic acid treatment of rats, mice and guinea pigs(1-4). Although those data suggested that under such conditions the vitamin probably plays a compensatory role similar to cortical hormones, other observations indicated that the similarity is not complete. It was shown that the protective action of the vitamin against cold stress was not present in adrenalectomized rats(5) and that ascorbic acid *per se* failed to alter the adrenal cholesterol level, the blood leukocyte pattern, or the blood glucose levels(4). Further observations indicated that ascorbic acid is capable of enhancing the gluconeogenic activity of small or large doses of cortisone in adrenalectomized mice. These observations suggest a "synergism" between cortisone and ascorbic acid(6), since the vitamin *per se* does not alter the liver glycogen content(4).

The present communication presents data indicating that ascorbic acid treatment of rats prolongs the leukocytic effects of injected cortisone or of endogenously secreted cortical hormones (induced by ACTH).

Materials and methods. Female Wistar rats weighing about 205 g were used in 2

* This study was aided by a grant from the National Institutes of Health. The Cortone was supplied through the courtesy of Dr. E. Alpert, Merck and Co.; ACTH through the courtesy of Dr. I. Bunding, Armour and Co.; and Vit. C, injectable, through the courtesy of Dr. R. A. Lewis, Hoffmann-La Roche.

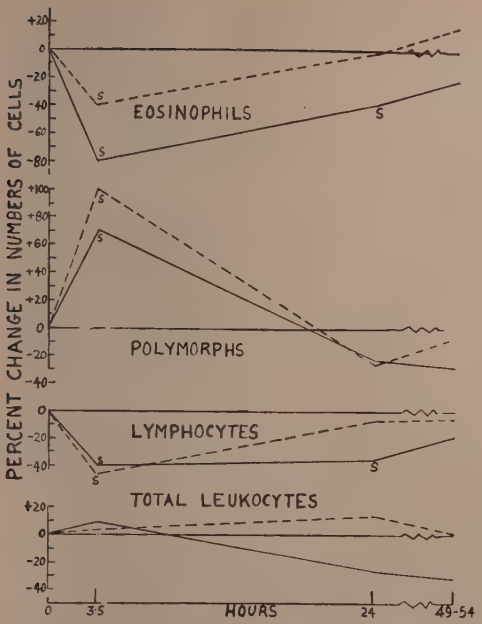

FIG. 1. Leukocyte response to cortisone in adrenex. Rats treated with ascorbic acid (——) or with saline (– – –). s = M.D. from count at 0 hr statistically significant.

experiments. For Exp. I the rats were bilaterally adrenalectomized (ether anesthesia) at least 2 weeks prior to use and received Purina Laboratory Chow and 0.9% NaCl solution *ad libitum*. For Exp. II adrenal-demedullated rats were used. The adrenals were enucleated (ether anesthesia) after the technic of Greep and Deane(7), at least 30 days prior to experimentation. Following operation, the animals received Chow and 0.9% NaCl for one week, and were then transferred to Chow and tap water. In both experiments tail blood was collected from all animals under nembutal anesthesia. The diluting fluid (eosin and methylene blue) was modified from that of Randolph(8). Total leukocytes, lymphocyte:polymorph ratio, and total eosinophils were determined by Randolph's method(8).

Experiments and results. Exp. I. Two groups of 8 adrenalectomized rats each were used. Group I was treated with 100 mg of ascorbic acid (Na ascorbate, Roche) per 100 g body weight by intraperitoneal injection. Fifteen minutes later these animals were injected subcutaneously with cortisone (Cortone), 2 mg per 100 g body weight. Group II rats received intraperitoneal injections of 0.85% NaCl, followed by the same dose of cortisone. Blood was collected from the tails of all animals (nembutalized) at the following times: a) just before the inj., *i.e.* at 0 hr; b) 3½ hr following inj. of cortisone; c) 24 hr following inj.; and d) 49-54 hr following inj.

The data from this experiment are presented in Fig. 1, which presents the percent change of the numbers of circulating cells from levels at 0 hr. Statistical analysis was previously carried out on the absolute numbers of cells and the Mean Differences ± S.E. from the count at 0 hr were calculated. Statistically significant differences are indicated by the "S" in the Figure. No marked change was observed in the total leukocyte levels. The lymphocytes showed significant decreases in both groups of animals at the end of 3½ hr following cortisone ($P < 0.01$ in both groups). The lymphopenia persisted in the Group I animals at 24 hr ($P < 0.05$) but not in the Group II animals. The levels of lymphocytes were not significantly different from base levels by 49-54 hr.

The polymorphonuclear leukocytes are significantly elevated ($P < 0.01$ in both groups) in both groups of rats at the end of 3½ hr, but not at 24 or 49-54 hr. The eosinophil levels are significantly decreased in both groups of animals at the end of 3½ hours after injection ($P < 0.01$ in Group I, $P < 0.05$ in group II). The eosinopenia persisted in the Group I rats at 24 hours ($P < 0.05$) but not in the Group II rats. The levels are back to baseline values at 49-54 hr. The data demonstrate that the eosinopenic and lymphopenic actions of *cortisone* persist for a longer time in animals which received ascorbic acid plus cortisone than in control animals. While it was possible to detect an intensification of the effect of small doses of cortisone by ascorbic acid in mice(6), such a phenomenon is not observed here. It is possible that the dose of cortisone was so high in this case that the presence of the vitamin was of no marked consequence at 3½ hours, but served to prolong the effects

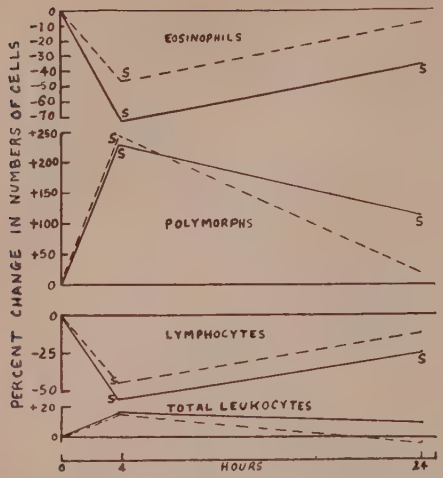

FIG. 2. Leukocyte response to ACTH in adrenal-demedullated rats treated with ascorbic acid (——) or with saline (– – –). S = M.D. from count at 0 hr statistically significant.

of the hormone. The effect noted here is not due to the vitamin independently since, as stated before, ascorbic acid does not alter the leukocyte pattern. The effect is due to a joint action of ascorbic acid and cortisone.

Exp. II was conducted on 2 groups of 6 adrenal-demedullated rats each. Group I rats received intraperitoneally 100 mg of ascorbic acid per 100 g body weight. Fifteen minutes later these animals were injected subcutaneously with 0.5 mg of ACTH per 100 g body weight. Group II rats received 0.85% saline (instead of ascorbic acid) and ACTH. Tail blood was examined at the following times: a) just before the inj. (*i.e.* at 0 hr); b) 3½ hr to 4 hr after ACTH inj.; and c) 24 hr after ACTH inj. The data are presented as percent change in numbers of circulating cells in Fig. 2.

No marked change was observable in total leukocyte counts. The lymphocyte levels significantly decreased ($P < 0.01$) in both groups of rats at 3½ to 4 hr after ACTH. The lymphopenia persisted at 24 hours in the Group I ($P < 0.05$) rats, but not in the Group II rats. The polymorphonuclear leukocyte levels of both groups increased at 3½ to 4 hours ($P < 0.01$) after ACTH. This persisted in the Group I rats ($P < 0.01$) at the 24-hour count, but not in the controls. The circulating number of eosinophils significantly decreased in both groups ($P < 0.05$) at 3½ to 4 hours after ACTH, persisted in the ascorbic acid + ACTH group ($P < 0.05$) but not in the control group. Isolated counts showed that the cells were back to baseline levels within 50 hours after ACTH injection. The data established that the hematologic effects of ACTH in adrenal-demedullated rats are prolonged when animals received simultaneous ascorbic acid treatment.

Discussion. The data of the present study indicate that ascorbic acid treatment prolongs the hematologic effects of exogenous and of endogenous cortical hormones. These prolonged effects suggest that detectable amounts of hormones are probably present for a longer period in the animals which received ascorbic acid. While the mechanism of maintenance of cortical hormone levels is not known, certain data were obtained in this laboratory indicating that it may be due to diminution of excretion or diminution of inactivation of the hormones in the presence of the vitamin. It was observed that 17-ketosteroid excretion in the 24-hour urine was significantly decreased in cortisone + ascorbic acid-treated female adrenalectomized rats (unpublished data). This study is being expanded.

With these considerations in mind it is possible to describe the relationship of ascorbic acid to pituitary-adrenal axis activity. Ascorbic acid blocks the activation of the axis to non-specific stressors. The exact locus of this interference is not known, but it is not between the pituitary and the adrenal cortex, *i.e.*, the vitamin does not block the action of ACTH. Furthermore, it is not at the level between the adrenal cortex and the periphery, since ascorbic acid does not block cortisone action(4). Indeed, it has been shown that the vitamin may enhance cortisone-gluconeogenesis(6). The vitamin does not have a direct effect on the leukocyte pattern, or on gluconeogenesis (3,4). It may be added that the vitamin is capable of blocking the activation of the axis without having any demonstrable lytic action on non-specific stressors; it has been shown that ascorbic acid fails to inactivate injected epinephrin or, to block directly the actions of trauma(4).

In view of the above it seems likely that ascorbic acid blocks the activation of the axis by inhibiting in some manner the activation of the pituitary. Although the exact details of pituitary activation are controversial, it has been generally accepted that the titer of circulating cortical steroids plays an important role in the regulation of pituitary-adrenotrophic activity. Sayers and Sayers (9) have shown that high titers of cortical hormone in the blood depress pituitary ACTH release whereas low titers increase this activity of the pituitary. Data were obtained in this laboratory indicating that ascorbic acid probably decreases the inactivation of cortical hormones. (It was observed that the excretion of 17-ketosteroid metabolites of cortisone in adrenalectomized female rats is significantly decreased by simultaneous ascorbic acid treatment). By the above process the maintained levels of circulating cortical hormones would tend to depress pituitary-adrenotrophic function. Under such circumstances non-specific stressors fail to activate the pituitary-adrenal axis.

This proposed mechanism does not include the suggestion that ascorbic acid is necessary for the secretory activity of the gland. Previous observations(10-12) indicated that the adrenal of the scorbutic animal is capable of producing cortical hormones in response to ACTH. The decreased adrenal hormonal effects observed by Giroud in scurvy(13) were probably not due to a decrease of production of hormones, but to an absence of the post-adrenal synergism with ascorbic acid in scurvy. McKee *et al.*(14), for instance, observed that the gluconeogenic action of injected cortical hormones is diminished in scurvy. While Dugal suggested that: a) ascorbic acid *per se* may activate the pituitary adrenal axis(15), or b) that it synergizes with ACTH(16), data to that effect have not been observed in this laboratory. It may be added that in the later paper Dugal stated the vitamin *per se* does not activate the axis.

Summary. The influence of ascorbic acid treatment on the hematologic effects of cortisone and of ACTH was investigated. The vitamin (plus hormones) prolongs these effects beyond that in animals which receive saline + hormones. These data suggest that the circulating titer of cortical hormone is maintained for long periods by vitamin treatment. This observation is discussed in relation to previous findings, and a mechanism proposed for the relationship of ascorbic acid to pituitary-adrenal activity.

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Received April 7, 1952. P.S.E.B.M., 1952, v80.

Effect of X-rays on Thymocytes and Its Modification by Cysteine. (19534)

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That many of the biological effects of X-irradiation are diminished by lack of oxygen or the addition of chemicals such as cysteine seems firmly established(1). The mechanism of the protection is poorly understood, however. It has been inferred from the radiochemistry of water that short-lived, reactive intermediates, primarily oxidants, form in the aqueous environment of the cell and of the intracellular elements. Anoxia may act by decreasing the formation of such intermediates and cysteine by competing for them. On the other hand, there is the possibility that these factors may also protect by altering the quality or quantity of the biological targets, *e.g.*, enzymes or genes(2). In the former instance, the biological effectiveness of the radiation would be decreased, while in the latter case, the radiosensitivity of the biological system would be diminished. There is, of course, no compelling reason to believe that the mode of action is identical in all living systems or indeed in a specific system under different conditions.

As part of a study of protective mechanisms, we are investigating the effects of cysteine, anoxia and related factors on irradiated suspensions of thymic cells. It was believed that this simple system would make possible a more precise evaluation of the protective phenomena. The suitability of thymic cells for this purpose was apparent from the work of Schrek(3). The thymic cell response to X-radiation and certain of the characteristics of its modification by cysteine will be described in the present report.

Methods. Inbred rabbits of either sex weighing about 1.5 kg were anesthetized with nembutal. The thymus was removed aseptically, dissected free of fat, and minced with scissors on a sterile watch glass. The mince was then placed on a monel metal screen (No. 80 mesh) in a Seitz filter and washed with 10 cc Ringer solution buffered at pH 7.4 with M/15 Na_2HPO_4 and NaH_2PO_4. The filtrate was centrifuged for 5 minutes at 600 g, the supernate discarded and the packed cells resuspended in a solution containing equal volumes of homologous serum and phosphate buffered Ringer solution. Similar cell concentrations were readily achieved from one experiment to another by making a 1:20 dilution by volume of the packed cells with the serum-Ringer solution. The stock suspension, consisting for the most part of small cells with large nuclei and scanty cytoplasm, was divided immediately into 2 cc aliquots placed in 15 cc conical test tubes. Three cc of the serum-Ringer diluent containing cysteine HCl neutralized with sodium hydroxide or the equivalent of sodium chloride were then added. Varying amounts of cysteine were used, the concentrations ranging from 3.2 to 19.2 mM. These samples contained approximately 100 mg of cells with a concentration of 40000 to 60000 cells per cu mm. About 75% of the cells were viable as determined by eosin staining. In some experiments, the serum-Ringer solution containing cysteine or sodium chloride was added after irradiation. Although the cell concentration during irradiation was more than doubled by this procedure, as will be shown later radiosensitivity was not affected. At least 4 aliquots were prepared in each experiment, 2 containing cysteine and 2 sodium chloride. Only one of each pair was exposed to the X-rays. All of the tubes were incubated with frequent shaking in a water bath at 37°C for varying periods of time prior to irradiation. The X-radiation factors were 200 kv, 15 ma, 0.25-mm Cu and 3-mm Bakelite filters, 32.5 cm target distance, 0.76-mm Cu half value layer and 100 r per minute dose rate. The total radiation dose ranged from 50 to 2000 r. In a few experiments the effects of irradiation at 100 and 500 r per minute were compared. After the exposure, two 0.2 cc aliquots were pipetted from each of the test tubes into 10 cc sterile culture tubes, tightly stoppered, placed at a 25° angle in a water bath at 37°C and shaken at a rate of 125 times per minute. Cell counts were

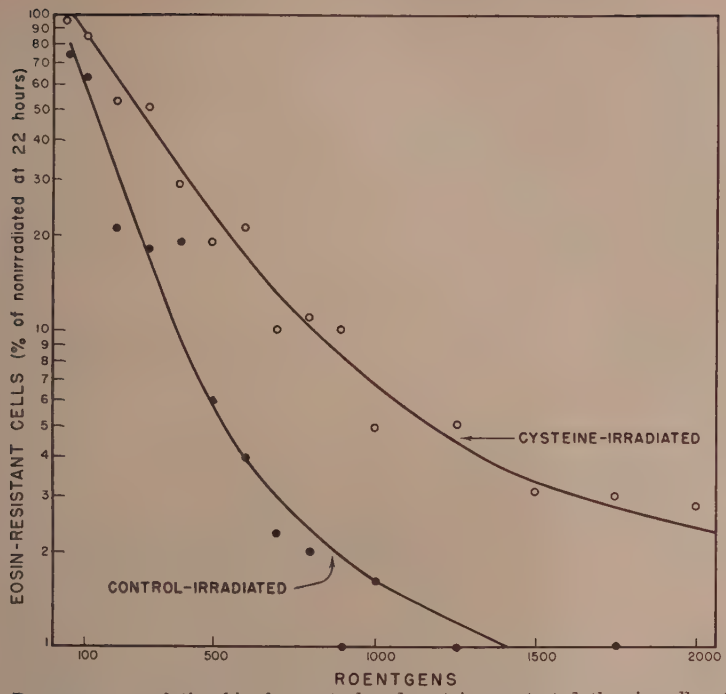

FIG. 1. Dose-response relationship for control and cysteine protected thymic cells. (Cysteine added to cell suspensions 15 min before X-irradiation, final conc. 19.2 mM).

generally made 22 hours after irradiation. Tyrode's solution (3.8 cc) containing 1% Eosin Y (National Aniline) was added to the 0.2 cc aliquot of the cell suspension. The mixture was shaken for 2 minutes, a drop was placed in a Spencer Bright Line hemacytometer and a count was made of the number of unstained cells in the central square of the chamber (0.1 cu mm) using a binocular microscope and a magnification of 270. Two hundred to 300 unstained cells, exclusive of erythrocytes, were counted. Occasional tests were made to rule out the presence of bacteria.

Unstained cells are assumed to be viable since they exhibit motility, protoplasmic streaming and pseudopod extension when cultured(4). On the other hand, quiescent cells, which resemble indistinctly granular spheres and manifest varying degrees of molecular vacuolization stain with eosin. Since thymic cells prepared in this manner almost never divide, population reduction due to cellular senescence is a constant factor, 50 $\pm$ 9% of the control cells staining with eosin at 22 hours. Cysteine in the concentrations employed did not alter the viability of the nonirradiated cells. The number of eosin-resistant cells in the irradiated suspensions was expressed as a percentage of the nonirradiated population for each thymic preparation at 22 hours. In a few experiments only the serum-Ringer medium was irradiated with and without cysteine and the thymic cells were added immediately afterward. In other studies cell preparations were centrifuged at 600 g for one minute immediately before irradiation. The packed cells with the supernate present were then exposed to X-rays, after which they were resuspended in the same fluid.

Results. X-radiation decreases the life span of rabbit thymocytes *in vitro,* the percentage of surviving cells (eosin resistant) decreasing exponentially with increasing radiation dosage. As may be seen in Fig. 1, the 22-hour LD_{50} is 125 r and definite effects appear with 50 r, a dosage which is similar to the threshold

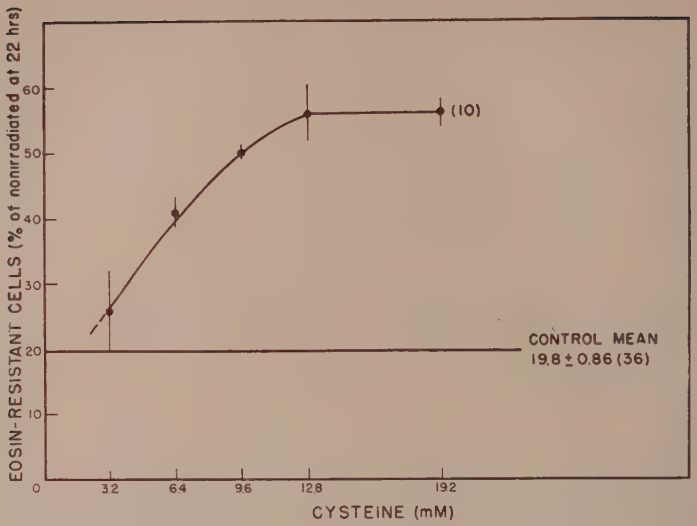

FIG. 2. Relationship between cysteine concentration and radiation effect (200 r). (Each value represents the mean of 3 experiments unless designated otherwise. Vertical lines refer to standard error of mean.)

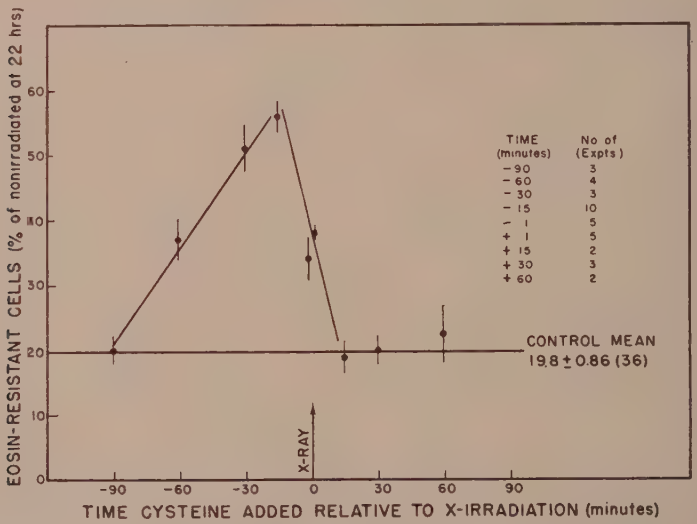

FIG. 3. Time course of the cysteine protection against 200 r. (Cysteine added at designated times to make final concentration 19.2 mM. Vertical lines refer to standard error of mean.)

for lymphopenia following whole body irradiation. Comparable results were reported previously (3). The thymic cell response is identical with dosage rates of 100 and 500 r per minute and cell concentrations of 40000 and 80000 per cu mm. Cysteine displaces the radiation dose-response curve by a constant percentage over a wide dosage range (Fig. 1). In effect, cysteine appears to cancel 0.53 r (σ 0.019 r) for each roentgen that is delivered to the cell suspension.

The degree of protection is dependent upon the concentration of cysteine as well as upon the time of its addition relative to the period

TABLE I. Cell Survival Following Irradiation of the Medium or of Packed Cells.

Group	Roentgens	No. of exp.	Eosin-resistant cells at 22 hr, % of nonirradiated
	A. Medium irradiated		
Control	500	1	65
"	1000	1	58
Cysteine*	1000	1	95
Control	5000†	1	49
Cysteine	5000	1	97
	B. Packed cells		
Air	200	3	39 ± 3.6‡
Air-cysteine	200	3	50 ± 3.9
Oxygen	200	4	24 ± 1.4
" -cysteine	200	2	50 ± 2.6

* Final conc. 19.2 mM.

† Dose rate 500 r/min; other exposures at 100 r/min.

‡ Estimated standard error of the mean.

of irradiation. The minimal effective concentration is approximately 6.4 mM, and maximal protection is noted with 12.8 and 19.2 mM, the latter being the maximally tolerated dose. Since our analyses showed that cysteine is equally distributed between cells and fluid, these concentrations correspond to doses ranging from 0.4 to 2.3 mg of cysteine per g of cells. The relationship between the cysteine dose and the radiation effect is presented in Fig. 2. The time course of cysteine protection is given in Fig. 3 where it will be seen that the survival of irradiated thymocytes is increased to the same degree whether cysteine is added one minute before or after exposure to 200 r. Addition 15 to 30 minutes before irradiation is optimal, however. Significant protection is also obtained when the amino acid is introduced into the suspension 60 minutes before the exposure, while its addition 90 minutes before or 15, 30, or 60 minutes after the exposure does not enhance cell survival.

Irradiated samples of the serum-Ringer solution are slightly toxic to thymic cells. Toxicity of the irradiated medium is prevented by the addition of cysteine. The factors responsible must persist for only a brief interval since the effect changes only slightly with increasing radiation dosage. Of interest is the finding that thymocytes packed by centrifugation are considerably less sensitive than cells suspended in serum-Ringer solution, even though the supernate is present during the exposure. The survival of such packed cells is not increased significantly by cysteine. On the other hand, cells equilibrated with oxygen before centrifugation appear to be as sensitive as cells in suspension and, in contrast to the results noted above, are readily protected (Table I).

A few nonirradiated and irradiated thymic cell preparations were incubated at 37°C on a warm stage microscope and studied cinematographically. Control cells and cells to which cysteine was added were essentially similar in their appearance and activity. Although there was no immediate difference between the irradiated controls and the cysteine treated cells, preparations of the latter contained many motile forms at 24 hours, while those of the former were predominantly quiescent. Such quiescent cells stained with eosin.

Discussion. A given amount of cysteine accounts for a rather constant percentage of the biological effect of irradiation over a wide dose range. This can be explained equally well by postulating an interaction with either the radiation or its toxic intermediates or with the biological system. A similar proportionality and degree of protection have been observed in mice for lymphopenia, granulocytopenia, splenic involution and lethality(5). The relatively uniform protection against a number of radiation sequelae points to a true dose reduction and suggests that cysteine alters a common pathway. The report(6) that glutathione pretreatment does not affect splenic and thymic involution following X-irradiation is not at variance with these findings since the anticipated difference in organ weights between 800 and 600 r, which represents the degree of protection by glutathione from survival data(7), is only about 5%(8).

Although there is a clear relationship between cysteine dosage and its effectiveness at a given time, protection of thymic cells *in vitro* is not a simple function of the sulfhydryl level during exposure. Equivalent sulfhydryl concentrations are found, within 15 minutes, in cells and ambient fluid; the levels decline at a constant rate (30% in 90 minutes). Addi-

tion of 9.6 mM of cysteine 15 minutes before irradiation confers definite protection, while the addition of twice this amount 90 minutes before is not effective, although the sulfhydryl concentrations at the time of exposure are comparable. This difference is probably not a result of the decreased ratio of reduced to oxidized sulfhydryl with time. It may be a consequence of exhaustion of essential precursors, secondary reactions, or redistribution of cysteine within the cell. Under these conditions, the relative effectiveness with time of administration (Fig. 3) might depend upon cysteine dosage. An optimal reaction time is also indicated since protection decreases as the irradiation period is approached. The postirradiation effect, as well as that of pretreatment, may be a result of the reversal of a chain of radiation action, reconstitution or replacement of an injured target, or substitution of an alternative metabolic pathway. Persistence of toxic substances in the medium can account for only part of the postirradiation effect.

It is well to recall that the time course of protection against acute radiation lethality follows a somewhat different pattern in the intact animal(9). The optimal time of administration in rats and mice is immediately before irradiation; there is no after effect. The apparent discrepancy may be a consequence of differences in the kinetics of reactions with cysteine and in the time constants for development of irreversible injury. The thymic cell suspension is a relatively sluggish, non-dividing system, in which oxygen tension is rate limiting for the disappearance of cysteine, presumably by oxidation(10). On the other hand, the tissues that appear to be causally related to lethality of the animal are those with the most rapid cell turnover.

The apparently anomalous finding that packed cells are relatively radio-resistant and not affected significantly by cysteine may be attributed to their hypoxic state. Results of other experiments now in progress are consistent with these observations and indicate that removal of oxygen from the thymic cell suspension is as effective as cysteine but that the two are not additive. Although cysteine has been shown to increase the survival of aerobically-grown *E. coli* irradiated in an oxygen-free phosphate buffer, anaerobically-grown bacteria resemble thymic cells in the lack of additivity(11,12). These considerations suggest perhaps that cysteine may act by diminishing availability of oxygen in the biological system. If this is true, less complete oxygen deprivation and minimal amounts of the amino acid may be additive in their effects on thymic cells. This does not imply, however, that oxygen diminution *per se* is the decisive event in the protection of thymic cells by cysteine. There is, in fact, reason to believe that other events must ensue, since protection appears to be dependent upon temperature during the early postirradiation period.

Summary. Sensitivity of rabbit thymocytes to X-irradiation *in vitro* is decreased by a constant factor over a wide dosage range when cysteine is added to the suspension. Protection depends upon cysteine concentration and time of administration but is not a simple function of the sulfhydryl level. There is a definite postirradiation effect of cysteine which can be accounted for only in part by the persistence of toxic substances in the medium. The relative resistance of packed cells and the failure of cysteine to protect them may be a consequence of hypoxia. It is suggested that the effect of cysteine on irradiated thymic cell suspensions may be a result of competition with cell substrates for oxygen, but that the binding of oxygen *per se* is not necessarily the decisive event in the protection. The results are discussed in connection with related observations in the intact animal.

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Received April 7, 1952. P.S.E.B.M., 1952, v80.

Effect on Joint Permeability of Adrenal Cortex Extract,* ACTH, Cortisone Hydroxy-phenyl Cinchoninic Acid and Hyaluronidase. (19535)

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Recent experimental and clinical studies have indicated a functional relationship between the mesenchymal tissues, the anterior pituitary gland and the adrenal cortex. The sequence of events in this relationship indicates that under conditions of stress, augmented adrenocorticotropic hormone (ACTH) production stimulates the adrenal cortex to release corticoid hormones. The corticoid hormones influence, in some way, the integrity of the connective tissues or ground substance. However, the changes in function and structure of the mesenchymal tissues which occur under the influence of these hormones are still not completely understood. It has been postulated that the mechanism of action of ACTH and cortisone is related in part to inhibition of the enzyme hyaluronidase and to a resulting decrease of membrane permeability(1-4). Furthermore, it has been hypothesized that the above mentioned hormones may exert their beneficial effects in the collagen diseases in part at least through this action on permeability(5,6).

In an attempt to determine whether joint permeability of rabbits is altered by injection of these hormones and related substances, a method was used which is similar to that reported by Seifter *et al.*(4). It consists of injecting phenolsulfonephthalein solution (PSP) into the knee joint of a rabbit under nembutal anesthesia and determining the rate of its excretion in the urine. The following procedure was carried out:

The bladder of the anesthetized rabbit was washed with fresh 10 cc portions of tap water until the sample withdrawn was clear. After waiting 10 minutes the bladder was washed again; a 10 cc portion of water was then introduced and withdrawn from the bladder to be used as the blank specimen in the photocolorimetric determination of PSP. Twenty-five hundredths cc (1500 μg) of PSP was then injected into the knee joint of the rabbit. Care was taken to ensure that the PSP was injected into the joint cavity and that hemorrhage and undue trauma were not produced. After 5 minutes the bladder was washed and a sample taken in the described manner. Similar samples were obtained at 10-minute intervals throughout the remainder of the experiment, which lasted 135 minutes. The time of appearance of PSP in the urine was noted, and the concentration of dye measured in each sample, using a Lumetron photoelectric colorimeter. After the above control studies were performed separate series

* We are indebted to Dr. David Klein, Wilson Laboratories, Chicago, for the cortical adrenal extract.

TABLE I. Route and Time of Administration, Number and Total Dose of Substances Used.

Drug	Animal No.	Route	Time of admin. before dye, hr	No. of doses	Total dose
Cortisone	1	I.M.	1	1	10 mg/kg
	2	"	18, 12, 1	3	15 mg/kg
	3	"	19, 1	2	20 mg/kg
	4	"	44, 25, 17, 2	4	25 mg/kg
ACE	1	"	1.5	1	1 cc
	2	"	4, 2	2	2 cc
	3	I.V.	.75	1	1 cc
	4	I.M. & I.V.	15, 1	2	2 cc
HPC	1	Oral	24, 2	2	200 mg/kg
	2	"	24, 2	2	200 mg/kg
	3	"	24, 2	2	200 mg/kg
ACTH	1	I.P.	12, 1	2	4 mg/kg
	2	"	48, 24, 12, 1	4	8 mg/kg
	3	"	30, 18, 12, 6	4	12 mg/kg
	4	"	30, 18, 12, 6	4	14 mg/kg
Hyaluronidase	1	Into joint	With PSP	1	100 TRU*
	2	" "	" "	1	100 TRU*
	3	" "	" "	1	100 TRU*
ACTH + hyaluronidase	1	I.P. : joint	24,† 18, 13, 7, 1	5	38 mg : 100 TRU
	2	" "	25, 19, 13, 7, 1	5	
	3	" "	25, 19, 13, 7, 1	5	40 mg : 100 TRU
	4	" "	25, 19, 13, 7, 1	5	

* TRU = Turbidity reducing units.
† ACTH injection times; hyaluronidase was given with PSP.

of experiments were conducted to determine the influence of hyaluronidase, of ACE (Adrenal cortical extract, prepared by Wilson Laboratories), of 11-dehydro-17-hydroxy-corticosterone (Cortone Acetate, Merck) of adrenocorticotropic hormone (ACTH, Armour) and of 2 hydroxy-phenyl cinchoninic acid (HPC)(7,8) upon the permeability of the rabbit's synovial membrane to PSP, as revealed by the rate of excretion of this substance in the urine after injection into the knee joint. Cortisone, ACE, and ACTH were given intramuscularly, intraperitoneally, or intravenously at varying intervals before the dye was injected. Because of its insolubility in water, HPC (Lilly) was administered in a suspension by stomach tube before PSP was given. Hyaluronidase was injected directly into the knee joint. The substances were administered in the dosage and at the intervals shown in Table I.

Results. The results are shown graphically in Fig. 1-5. The mean values for PSP excretion by control animals are labeled on all the graphs. In all 10 control rabbits, the dye appeared within 5 minutes and reached a maximum concentration in either the 25th or 35th minute sample. The 135th minute samples contained an average of 18 γ (μg) of PSP. The average of the total dose excreted by the controls in the 135 minutes was 65% with a range of 57 to 77% (Fig. 1). Although the standard error (SE) has little statistical significance when computed on such a small series of animals, the ± 2 SE is used here to indicate the scatter of the control values.

The mean rate of PSP excretion in the cortisone-treated group is indicated in Fig. 1. The mean total excretion of PSP in this series was 65% with a range of 61-72%. It will be observed that the mean total excretion by these animals was influenced little, if at all, by the administration of cortisone.

Whitehead and Darley(9) had obtained suggestive clinical results in a series of 8 cases of rheumatoid arthritis, with an extract of adrenal cortex prepared by the method of Swingle and Pfiffner(10). Accordingly, we studied the effect of ACE prepared by their method on the permeability of the knee joint to PSP. The adrenal cortex extract used in this series of experiments was prepared for us by Dr. David Klein, of Wilson Laboratories, Chicago, and was adjusted in strength so that

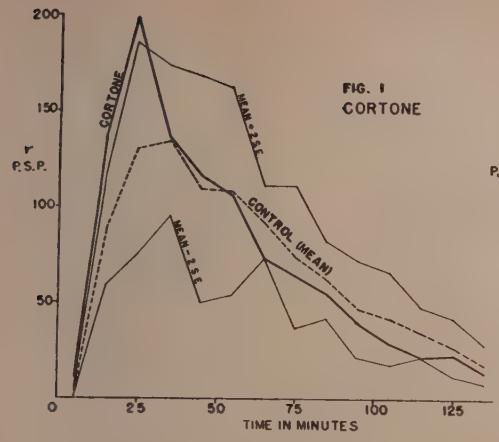
FIG. 1
CORTONE
CORTONE
MEAN ± 2 S.E.
CONTROL (MEAN)
MEAN ± 2 S.E.
γ P.S.P.
200
150
100
50
0
25
50
75
100
125
TIME IN MINUTES

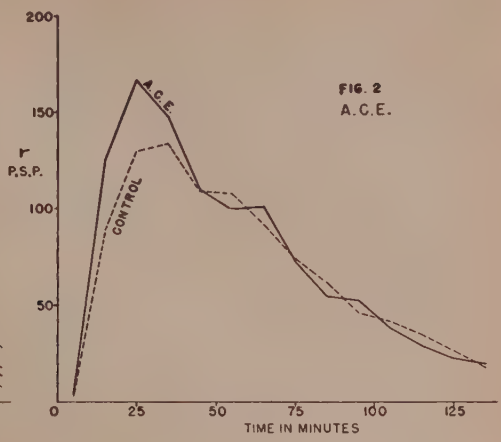
FIG. 2
A.C.E.
A.C.E.
CONTROL
γ P.S.P.
200
150
100
50
0
25
50
75
100
125
TIME IN MINUTES

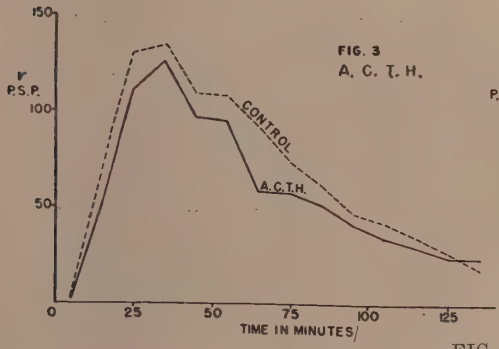
FIG. 3
A. C. T. H.
CONTROL
A.C.T.H.
γ P.S.P.
150
100
50
0
25
50
75
100
125
TIME IN MINUTES

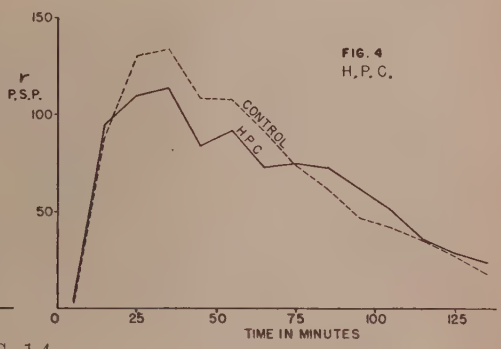
FIG. 4
H.P.C.
CONTROL
H.P.C.
γ P.S.P.
150
100
50
0
25
50
75
100
125
TIME IN MINUTES

FIG. 1-4.

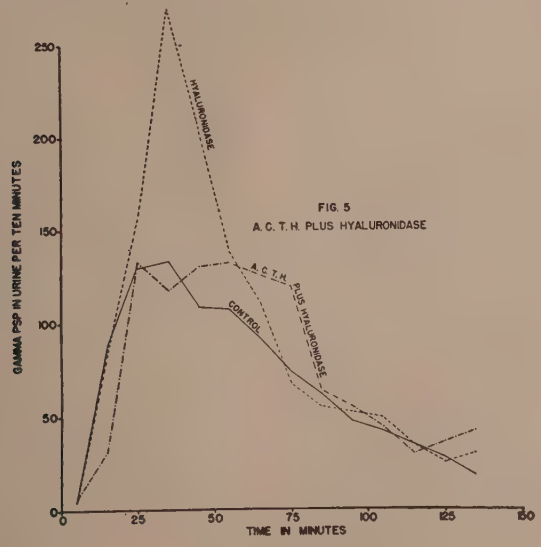
FIG. 5
A.C.T.H. PLUS HYALURONIDASE
HYALURONIDASE
A.C.T.H. PLUS HYALURONIDASE
CONTROL
GAMMA PSP IN URINE PER TEN MINUTES
250
200
150
100
50
0
25
50
75
100
125
150
TIME IN MINUTES

FIG. 5.

1 cc of extract was equivalent to 30 g of beef adrenal. We are greatly indebted to Dr. Klein for providing this product. The average excretion of PSP in the 4 animals treated with ACE is indicated in Fig. 2. The mean total PSP excretion was 62% with a range of from 59 to 68%. These results, therefore, provide little evidence that ACE had any influence on the permeability of the joint membrane and/or the kidney to PSP.

The influence of ACTH on the excretion of PSP is shown in Fig. 3. The mean total excretion was 54% with a range of from 45 to 61%. Of all the substances used in these experiments only ACTH may have had a slight depressant effect upon either joint or kidney permeability.

The results of the administration of HPC 100 mg/kg administered orally 24 and 2 hours prior to the PSP injection are shown in Fig. 4. The mean total excretion of PSP was 61%; range 57-64%.

Each of 3 rabbits was given 100 turbidity reducing units (TRU) of hyaluronidase (Alidase, Searle) dissolved in the 0.25 cc of PSP. This mixture was injected into the synovial capsule of the knee joint. The results of this experiment are illustrated in Fig. 5. The total excretion of PSP averaged 85%; range, 82-86%; this was a marked increase over control values. Apparently there is a latent period of approximately 25 minutes before the enzyme becomes effective, since the initial portions of the PSP excretion curves of the control and enzyme treated animals are practically superimposable for that period.

The effect of the combined administration of hyaluronidase and ACTH on the excretion of PSP is shown in Fig. 5. The ACTH was given intraperitoneally in the dose and at the intervals shown in Table I. The average excretion of PSP was 71% (range 69-74%). In these experiments ACTH appeared to counteract the effect of hyaluronidase upon the rate of excretion of PSP.

Discussion. The results secured in the present work indicate that normal joint permeability as determined by the PSP test is not decreased appreciably by adrenal cortex extract, cortisone acetate, ACTH, or HPC. The results obtained are confirmatory of those reported by several other investigators (2,11,15) using different technics. It seems possible to say unequivocally that adrenal cortex extract, ACTH or cortisone do not alter the permeability of the *normal* joint membrane or any other tissue that has been tested. On the contrary when permeability has been increased by hyaluronidase administration(3,4) or by inflammation(11) then the 11-oxysteroid hormones and ACTH tend to reduce the permeability toward the normal.

Whether permeability of the joint membrane to PSP reflects in any accurate fashion the normal state of this tissue is of course open to question. That ACTH or cortisone do not alter the permeability of the kidney to PSP has been determined by Paul *et al.*(12).

The possibility that a reduction in permeability is an important common denominator in the anti-inflammatory action exercised by cortisone and indirectly by ACTH has been emphasized by several writers(13-15). Moon *et al.*(14) observed that diapedesis, capillary hemorrhage, edema, hyperemia, and leucocytic infiltration induced by thermal injury were much less marked in cortisone treated animals. The resistance of the capillary walls is known to be increased by cortisone, and this may be a common factor in the reduction of each of the features in the inflammatory reaction. Whether the effect of these hormones is primarily on permeability or whether other factors are also prominently concerned in the anti-inflammatory action still remains undetermined.

Summary and conclusions. 1. Permeability studies were conducted on rabbits by injection of PSP into the joint and recovery of the dye from the urine. 2. Cortisone, ACTH, adrenal cortex extract and hydroxyphenylcinchoninic acid (HPC) given to normal rabbits failed to cause any significant alteration of synovial permeability. 3. ACTH given parenterally exhibited a significant antihyaluronidase effect. 4. We agree that the permeability of the joint membrane to PSP is not necessarily a measure of its permeability to normal body substances.

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Received April 21, 1952. P.S.E.B.M., 1952, v80.

Amino Acids of Turnip Yellow Mosaic Virus.*† (19536)

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A study has been undertaken in our laboratory of the adsorptive properties of the recently described crystalline turnip yellow mosaic virus(1) and of the nucleic acid derived therefrom. A complete analysis of the nucleic acid moiety of the virus has been published(2). The present communication contains the analysis of the protein prepared from the virus.

Experimental. The protein was liberated from the virus by treatment with 35% ethanol at room temperature at a neutral pH(1). The precipitated protein was washed free of nucleic acid. Suitable quantities of the protein were hydrolyzed and analyzed for amino acids by microbiological and paper chromatographic procedures which have been described in detail in a previous communication(3).

Results. Chromatographic findings. A typical 2-dimensional chromatogram prepared from a volume of acid hydrolysate corresponding to 1 mg of the original protein is shown in Fig. 1. Eighteen ninhydrin-reactive substances were detected. In addition to the usually occurring amino acids small amounts of constituents which have been *tentatively* identified as glucosamine (spot 2), α-amino-n-butyric acid (spot 6), and γ-aminobutyric acid (spot 11) were also observed. It is not certain whether the latter 3 substances are present as impurities or are actually constituents of the virus protein. Tryptophan was not detected because of destruction during acid hydrolysis, and the phenylalanine spot was obscured by the large spot produced by the leucines. The most noteworthy finding on the chromatograms were the large quantities of proline, serine, and threonine. The proportion of threonine was greater than that found in any protein previously examined in our

* The production of the virus was made possible by a grant from the Charles F. Kettering Foundation. The work was aided by a research grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

† The authors are indebted to Dr. Kenneth M. Smith of the Agricultural Research Council Plant Virus Research Unit, Molteno Institute, University of Cambridge, England, for making the virus preparation available to them and to Dr. Roy Markham of the same institution for helpful suggestions.

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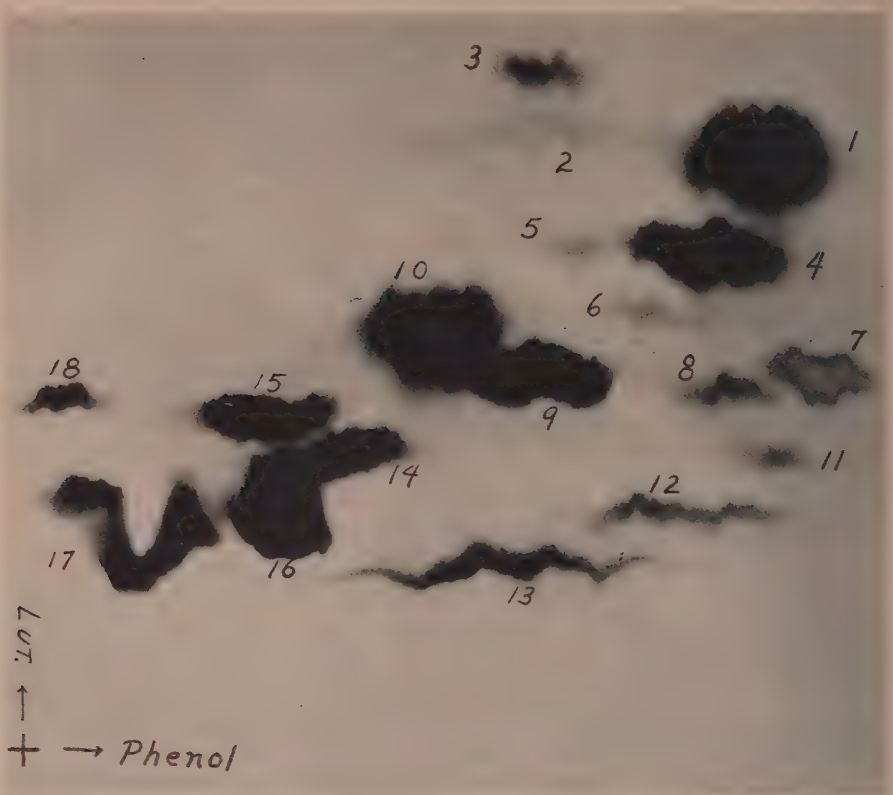

FIG. 1. Chromatogram of peroxide-treated hydrolysate of turnip yellow mosaic virus protein. Leucine and isoleucine, 1; glucosamine (?), 2; tyrosine, 3; valine, 4; methionine (methionine sulfone), 5; α-amino-n-butyric acid (?), 6; proline, 7; histidine, 8; alanine, 9; threonine, 10; γ-aminobutyric acid, 11; arginine, 12; lysine, 13; glycine, 14; serine, 15; glutamic acid, 16; aspartic acid, 17; cystine (cysteic acid), 18.

laboratory. The proline spot (spot 7) did not photograph with the same intensity as did the spots given by the other amino acids, since proline gives a yellow color while the other amino acids give colors ranging from blue to purple. No hydroxyproline was detected.

Microbiological determinations. From the results shown in Table I it is seen that almost all of the protein was accounted for by the analyses. There is a considerable excess of free basic groups over free carboxyl groups, making potentially available a number of loci which are capable of forming salt linkages with the phosphate groups of the nucleic acid. The presence of a large number of residues of serine and threonine suggests that the hydrogen bonding of the aliphatic hydroxyl groups may be important both in maintaining the internal structure of the protein and also in binding the protein to the nucleic acid.

Discussion. The finding of a large quantity of the aliphatic hydroxy amino acids in the protein moiety of the turnip yellow mosaic virus is of considerable interest since other proteins with interesting biological activities, human γ-globulin, pepsin, chymotrypsinogen, ribonuclease (see Tristram's compilation) (4), and Bence-Jones protein(3) have been found to have particularly high levels of these amino acids. In addition, it was found that the tobacco mosaic virus proteins(5) also contained relatively large amounts of serine and threonine. However, it is not possible at the present time to make any specific suggestions about

TABLE I. Amino Acid Composition of Protein of Turnip Yellow Mosaic Virus. All values are expressed on a moisture-free basis. Nitrogen content, 16 g/100 g protein.

	Amino acid			
	g/100 g protein	g residue/100 g protein	g N/100 g protein	Moles residue/10^6 g protein
Glycine	2.90	2.20	.54	38.6
Alanine	5.70	4.54	.91	64
Valine	8.45	7.14	1.01	72.1
Leucine	10.30	8.89	1.10	78.6
Isoleucine	10.80	9.31	1.15	82.4
Phenylalanine	3.80	3.39	.32	23
Proline	10.70	9.04	1.30	93
Tryptophan	1.14	1.04	.16	5.6
Cystine	2.50	2.31	.29	10.4
Methionine	2.85	2.50	.27	19.1
Glutamic acid	9.60	8.41	.92	65.2
Aspartic acid	5.10	4.41	.54	38.3
Histidine	2.10	1.86	.57	13.5
Lysine	10.70	9.40	2.05	73.2
Arginine	2.50	2.24	.80	14.4
Serine	8.10	6.71	1.08	77.1
Threonine	15.10	12.80	1.77	126.8
Tyrosine	2.45	2.20	.19	13.5
Amide NH_3	1.25	1.25	1.03	73.5
Total	116	99.6	16	982

Free basic groups, 101; free acid groups, 30; aliphatic hydroxy groups, 204.

the role that these amino acids might play in the biological activity of the proteins in which they are found in large amounts.

Summary. 1. The amino acids of the protein of crystalline turnip yellow mosaic virus were studied by paper chromatographic and microbiological technics. 2. Virtually all the protein was accounted for by microbiological assay for 18 amino acids performed on hydrolysates of the protein. 3. The protein was characterized by the presence of large amounts of serine, threonine, and proline. Hydroxyproline was not detected.

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Received April 23, 1952. P.S.E.B.M., 1952, v80.

Preparation of a Stable Form of Inulin for Tissue Analysis.* (19537)

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Direct analysis of tissue for inulin is essential for a correct evaluation of the volume distribution of this substance. Ross and Mokotoff(1) have described a method of analysis for muscle tissue using dilute alkali and heat to destroy the high inulinoid blank of this tissue. They were unable to obtain adequate recoveries of inulin added to muscle tissue by this method, unless the inulin had been previously treated with heat and dilute NaOH and reassayed before adding it to the muscle. Therefore, the analysis of the tissue of animals injected with the usually available inulin would not determine the total inulin present if this method were used. In studies involving the simultaneous determination of the total body inulin space and tissue distribution, it became necessary either to develop a method for tissue analysis which would not destroy the inulin, but, at the same time, would destroy the large inulinoid blank of muscle; or to develop a stable form of inulin for injection into these animals.

Table I indicates the results of treating inulin and muscle with various forms of digestion. From this, it is apparent that some inulin is destroyed by any procedure involving acid, alkali or heating. Inulin probably consists of at least 3 fractions—a heat or dilute acid labile one, a hot alkali labile one, and a hot alkali stable one. This is also suggested by the variation in molecular weight deter-

* Supported in part by a grant from the Heart Institute, National Institutes of Health, USPHS.

† Public Health Service Research Fellow of the NIH.

TABLE I. Recovery of Usually Available Inulin from Solutions and from Inulin-Muscle Mixtures.

Solution and treatment	% of original inulin remaining after treatment without added tissue	Inulinoid blank of muscle as % of recovered inulin
1. Water without heat	100%	300%
2. " + heat*	94	200
3. Dilute HCl without heat	95	200
4. " " + heat	92	200
5. " NaOH without heat	85	200
6. " " + heat	66	10

* Heated for 30 min in boiling water bath.
† .30 g of inulin/g of muscle; this corresponds to a plasma level of approximately 200 mg %.

TABLE II. Recovery of Stable Inulin after Treatment with Dilute NaOH and Heat.

.40 mg inulin and muscle added to water	Amt of inulin recovered, mg	% recovered
No muscle	.400, .397, .385	99
1 g muscle	.402, .350, .403, .381	96

minations with heating(2). Furthermore, the only method (No. 6) which satisfactorily reduces the inulinoid blank of muscle, also destroys the most inulin. Therefore, it is necessary to have a form of inulin for injection which will be stable in hot dilute alkali if analysis of tissues such as muscle is desired.

Purified stable inulin is prepared by first heating a 10% solution of chemically pure inulin with concentrated NaOH (5 ml NaOH for 100 ml of inulin solution) in a boiling water bath for 30 minutes. Further heating for 2 hours does not hydrolyze any more inulin. The resultant brown solution is then neutralized to pH 7 with concentrated HCl. The brown color is probably the result of carmelization of the hydrolyzed inulin. The stable inulin is then purified by repeated precipitation with 95% ethyl alcohol. About 5 volumes of alcohol are necessary for the immediate precipitation of one volume of 10% inulin solution. After precipitation, the solution is centrifuged, the supernatant discarded, and the precipitate is redissolved by adding about 75% of the original volume of distilled water and heating in a boiling water bath until solution occurs. The alcohol precipitation is then repeated. From 7 to 10 such precipitations are necessary to produce a pure white powder which gives a clear colorless solution when dissolved in distilled water.

Intraperitoneal injection of this inulin (0.55 g inulin/kg body weight) into weanling rats produced no observable ill effects and did not alter their rate of growth. This material was used for studies in nephrectomized dogs (0.25 g/kg body weight)(3) without any adverse effects. Typical recovery experiments are shown in Table II. Within the reproducibility of results possible with this method (±4%), we feel this represents complete recovery.

We have modified the method of Ross and Mokotoff(1) in two respects: first, by making our final volume, prior to digestion, to 35 ml instead of 50 ml in order to analyze smaller samples of tissue; second, in short-term experiments, by taking a specimen of tissue before the injection of inulin, for the blank determination in order to be able to treat the blank tissue and the experimental one in the same way.

Tissues such as skin and tendon which have only a small amount of inulinoid material in the blank, can be analyzed by extracting the tissue with a known amount of distilled water, after previously mincing the tissue. However, unless this mincing is done very carefully, the hot alkaline digesting method gives more consistent results.

Summary. 1. Current methods for the determination of inulin in tissues are not applicable for simultaneous studies of total body and individual tissue distribution of this substance. A form of inulin stable in hot alkali is required. 2. A procedure for the preparation of a purified form of inulin which is stable in hot, dilute NaOH is presented. 3. Toxicity and recovery experiments indicate this new form of inulin is satisfactory for the direct determination of the inulin content of the tissues of experimental animals following intravenous injection.

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Received April 24, 1952. P.S.E.B.M., 1952, v80.

An Apparatus for Airborne Infection of Mice. (19538)

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The continuous ventilation type of airborne infection apparatus first described by Wells (1) permits quantitative experimental infection of animals by inhalation of droplet nuclei bearing the panthogenic agent. The purpose of this report is to describe an apparatus of this type for the airborne infection of mice. This instrument† differs in the following respects from others of its kind which have been described in the literature(2-5): 1) It is small and compact, occupying less than 18 cubic feet of space, so that it can be placed on a laboratory bench or mounted on a table equipped with casters; it weighs less than 150 lb. 2) it is relatively inexpensive to build.

The infection chamber is 24″ in diameter and 14″ high, constructed of 21-gauge stainless steel, and covered with a removable UVA (impermeable to ultraviolet rays) plexiglass top, ½″ thick. (Fig. 1 and 2) It has a stainless steel bottom with a central opening onto which is soldered a short elbow pipe; on this pipe is slipped a 1″ i.d. "Tygon" hose. The hose is 6′ long and is attached at its other end to a nebulizer-venturi (N-V) apparatus. A removable steel baffle plate is located 1″ above the true bottom of the chamber; this baffle has a circular series of 24 round perforations ¾″ in diameter at 9½″ from its center. At 2″ above the baffle are located 5 projections from the wall of the chamber, and on these projections rests a removable basket-cage constructed of 12-mesh stainless steel wire mesh. The cage has a removable, flat, wire mesh top which can be rotated about a center post, and is divided by wire mesh partitions into five equal sectors so that five groups of mice (up to 20 mice each) can be kept separated in the cage and can be introduced and removed through a port located in the movable top. On the plexiglass top is mounted a 30-watt ultraviolet lamp‡ which consists of a horizontal circular tube (outside diameter of circle is 6″) with two vertical electrodes rising out of the chamber proper through a 3½″ diameter hole in the plexiglass. The electrodes are protected and the hole is covered by a round metal box, 4″ in diameter and 5″ high, with a flange at its

FIG. 2. Photograph of airborne infection apparatus (by Julian Carlile).

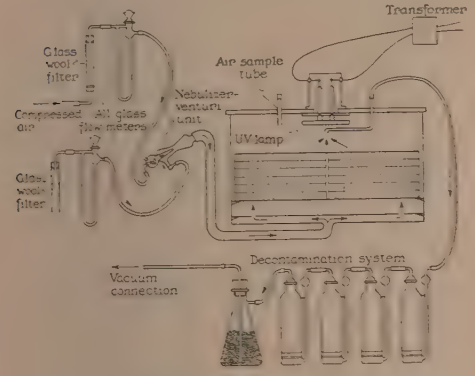

FIG. 1. A diagramatic representation (drawn by Ruth J. Mandelbaum).

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† Designed with the assistance of Josef Blum and constructed by him.

‡ Available from Hanovia Co., New York City.

open end through which it is tightly screwed with a rubber gasket onto the plexiglass top. Two other openings are provided for 7/16″ copper tubing with appropriate flanges and gaskets by which they are secured to the top, one for attachment of tubing for ventilating the chamber air into the decontamination system of gas washing bottles, the other for permitting removal of air samples to be tested for content of droplet nuclei containing microorganisms. The whole infection chamber is housed in a wooden box which is 28″ wide, 31″ long and 18″ high. As illustrated in Fig. 2, the front of the box has a recess 3″ deep which can be closed by a door hinged at the bottom. Bakelite supports are provided in this recess for 2 air-filters, 2 all-glass flow meters, and the nebulizer venturi apparatus. The plexiglass top is secured to the top of the chamber by means of 12 radially arranged screw clamps visible in Fig. 2.

The decontamination system consists of a series of 4 gas washing bottles, each having 350 ml capacity and a very coarse fritted glass filter disc.§ After the air from the infection chamber has been decontaminated (see *Experimental*) by passage through these bottles, it is passed through a dry flask filled with glass beads to remove aqueous droplets before evacuation into the house vacuum system. *The nebulizer-venturi unit,* as illustrated in Fig. 1 and 2, is an all-glass apparatus consisting of a "Vaponephrin" nebulizer annealed to two 25 ml Erlenmeyer flasks.‖ It is so designed that air under pressure from the house air supply is forced into the nebulizer, nebulizes the suspension therein, passes through the tapered exit neck of the nebulizer, is mixed, at a venturi opening at the point where the necks of the two flasks are annealed, with room air coming in through a side arm (½″ i.d.) near the base of the first flask, and, finally, passes through a 1″ internal diameter extension on the base of the second flask into the hose leading to the infection chamber. The suspension to be nebulized is introduced into the nebulizer through the opening provided in the commercially available unit; this can be performed aseptically by use of a syringe and needle. The opening can then be closed with a rubber stopper. *Measurements of airflow* are performed by means of two all-glass manometric type flow meters¶ containing dye-colored water. The compressed air admitted to the nebulizer is adjusted to flow at a rate of 5 liters per minute; this has been found to be adequate to yield 0.08 to 0.09 ml of very fine mist per minute. The main airflow is pushed into the system by atmospheric pressure because the whole interior of the apparatus, where the contaminated air is confined, is kept under negative pressure by the constant vacuum beyond the decontamination system. This main supply is adjusted to flow at 20 liters per minute. By varying the numbers of microorganisms in the suspension introduced into the nebulizer, the proportion of bacteria-bearing nuclei can be varied.

The following theoretical requirements have been fulfilled in the design of this apparatus: 1) The volume of air per unit time moving through the chamber is at least four-fold the volume of air per unit time breathed by 100 mice, the maximum number for which the apparatus was designed. (Total volume of air per minute passing through chamber is 25 liters, and the volume of air per minute breathed by 100 actively moving mice is 5 to 6 liters)(6). 2) The upward movement of air in the chamber is in excess of the settling velocities of all inhalable droplet nuclei. (The volume of the chamber is about 100 liters; the air moves through the chamber at the rate of 25 liters per min; and the height of the chamber is 14″. Therefore, the upward movement of air in the chamber is about 3.5″/min. This is greater than the settling velocity of the largest particles capable of inducing inhalation infection in experimental animals)(7).

Methods. The nebulizer fluid used in all experiments to be reported here was a five-fold dilution in distilled water of the Tween-albumin growth medium currently used for mycobacteria in this laboratory(8). The ad-

§ Corning Cat. No. 31750.

‖ Available from E. Machlett & Co., New York City.

¶ Corning Cat. No. 5960, calibrated with a wet-test meter.

dition of bovine serum albumin to suspending media for the airborne infection of rabbits with tubercle bacilli has already been shown to have a stabilizing influence on the viability and infectivity of these organisms(3). And, the five-fold dilution of the Tween-albumin medium gives a solution containing about 0.2% solids, which has been shown to yield aerosols composed of droplet nuclei of size suitable for inhalation(7). The mycobacteria employed in these experiments were cultivated in the above mentioned Tween-albumin liquid medium, for 3 days in the case of the non-pathogenic *M. ranae* and for 7 to 10 days in the case of the pathogenic bovine strain of tubercle bacillus, Vallée. Cultures of the *M. ranae* strain were composed predominantly of well dispersed single cells and were used without further treatment in the standardization experiments described below. Cultures of the Vallée strain contained a low proportion of single cells, being composed mostly of clumps containing 10 to 100 bacilli. Therefore, in one experiment to be reported here, a well dispersed suspension of this strain was prepared by a method like that described by Fenner(6). All experiments described here were 60-minute runs, with an added 10 minutes of airwashing to compensate for the delay in development of the ambient cloud in the infection chamber(4). An additional 20 minutes of airwashing, with the ultraviolet lamp on, was used when experiments involving pathogenic microorganisms were performed.

Experimental. Of primary import was the effectiveneness of the decontamination system of gas-washing bottles. It is obvious that experiments involving pathogenic microorganisms such as tubercle bacilli could not be suffered until it was demonstrated that microorganisms with similar properties would not be able to pass the decontamination system in significant numbers. Therefore, a flask of sterile medium (oleic acid albumin liquid medium) capable of supporting growth from individual bacterial cells of mycobacteria(8) was introduced at the end of the decontamination system so that air evacuated from the apparatus had to impinge onto the surface of the medium. The 4 gas washing bottles, each containing 300 ml of distilled water and 0.2 ml of tributyl phosphate (as antifoam agent), were autoclaved along with the flask containing glass beads and the flask of culture medium. A suspension of *M. ranae* containing approximately 10^8 bacterial cells per ml was employed in a routine 60-minute run. The flask of culture medium was then disconnected aseptically and incubated at 38°C. Several runs of this type were made and growth in the test medium never appeared before the fourth day of incubation. Previous studies had shown that at least 100 bacterial cells of this strain had to be inoculated into such a medium for growth to be evident before the fourth day. Thus the great majority of the 5×10^8 cells nebulized in such experiments was caught mechanically in this decontamination system.

These observations demonstrated that the decontamination system of this apparatus serves as an adequately effective mechanical remover of mycobacteria from contaminated air passing through the apparatus at the rate of 25 liters per min. Nevertheless, as an additional precaution, phenol in a final concentration of 2%, which is bactericidal for tubercle bacilli, was added to the distilled water in each gas washing bottle when these organisms were used in infection experiments.

In order to determine the percentage of fluid sprayed from the nebulizer which passes through the infection chamber as a cloud of droplet nuclei, phenol red was dissolved in a final concentration of 0.02% in the 5-fold dilution of Tween-albumin medium in distilled water, and this solution was used as the nebulizer fluid. The amounts of dye recovered from various parts of the apparatus after a run were determined colorimetrically after the methods of Rosebury(4). The amount of fluid nebulized was determined by weighing the nebulizer-venturi unit before and again after a run. The amount of fluid which impinged and dried on the exit neck of the nebulizer and in the distal flask of the unit was determined by washing out and discarding the nebulizer fluid remaining in the nebulizer after a run and then rinsing the entire nebulizer-venturi unit with dilute alkali. Table I shows the results of three such experiments. It is evident that under the conditions of these experiments approximately

TABLE I. Recovery of Clouds of Phenol Red from Nebulization of this Dye in the Airborne Infection Apparatus after Some Routine 60 Min Runs. Amounts of dye are expressed in milliliter equivalents of the original nebulizer fluid (NF) containing the dye in a final concentration of .02%.

	Exp. I	Exp. II	Exp. III
Wt of N-V unit + NF, ml:			
before run	77.1	77.7	77.7
after run	71.7	71.9	72.3
Fluid lost, ml	5.4	5.8	5.4
Impingement loss in N-F unit, ml	.60	.77	.64
A. Total nebulized, ml	4.80	5.03	4.76
Recovery from, ml:			
tubing from chamber to decontamination system + wash bottle #1	1.83	1.86	1.57
wash bottle #2	.54	.67	.78
wash bottle #3	.14	.16	.20
wash bottle #4	.05	.04	.06
B. Total recovered, ml	2.56	2.73	2.61
% recovery (B/A × 100%)	54	55	55

55% of the fluid lost from the N-V unit passes through the infection chamber as a cloud of droplet nuclei. The losses unaccounted for are probably attributable to impingement on the hose leading to the infection chamber and on the walls of the infection chamber itself.

An airborne infection experiment was performed in which 50 three-week-old white mice of the Rockefeller Swiss albino strain were exposed to the virulent bovine strain of tubercle bacillus, Vallée. The mice were equally distributed in the 5 sections of the basket cage. The number of viable tubercle bacilli in the nebulizer suspension was determined as 1.5 x 10^6/ml by plating out dilutions of this fluid on the oleic acid albumin agar medium(8). 5.8 ml of this suspension was nebulized in the one-hour run. From the experiments with phenol red, it can be estimated that approximately 0.05 ml of this suspension passed through the chamber each minute, diluted in 25 liters of air. Therefore, the concentration of droplet nuclei containing tubercle bacilli was approximately 2.5 x 10^3 per liter of ambient cloud in the infection chamber during the run. Each mouse is calculated to have inhaled about 4 liters/hour of this air. Therefore, each mouse, on the average, inhaled no more than 10,000 bacilli. The extent and uniformity of pulmonary pathology induced in this airborne infection apparatus was manifest when the mice were killed at 4 weeks after infection. There was no significant difference between the groups of mice infected in the different sections of the basket cage. Fig. 3 illustrates the appearance of the lungs and spleens of 5 unselected mice from one unselected section.

Another experiment was performed in which 77 mice were exposed in this apparatus in the same fashion except that an untreated Tween-albumin culture of the fully grown Vallée strain, diluted with 4 volumes of distilled water, was used as the nebulizer suspension. Suffice it to relate that, although such a suspension contains only a small proportion of well dispensed bacilli, it was possible to induce generalized pulmonary tuberculosis in all the exposed mice. Indeed, all of 10 mice sacrificed at 14 days after infection (2 occupants of each section of the basket cage) showed grossly visible pulmonary lesions, some of barely visible size. And, at 4 weeks after infection, the lungs of all of 15 mice killed at that time manifested widespread pulmonary consolidation, confirming the antemortem observation that all mice surviving at this time had obviously impaired respiratory function.

Comment. Two types of apparatus for the airborne infection of experimental animals have been described. In the first kind the animals are exposed in a closed chamber to a mist of aqueous droplets bearing pathogenic agents. It was this type that Glover used(9) when he successfully induced airborne infection of mice with tubercle bacilli. The second type permits quantitatively controllable infection by droplet nuclei, bearing the pathogenic agents, and delivered at a uniform rate to a measured air supply which ventilates the infection chamber at a constant rate. The advantages of the second type over the first have repeatedly been emphasized. It has been demonstrated that in this second type of apparatus it is possible to control the numbers of microorganisms inhaled by experimental animals exposed to droplet nuclei containing the microorganisms as single cells or as very small clumps, provided the number of micro-

FIG. 3. Photograph of the lungs and spleens of Swiss mice of the Rockefeller Institute strain, exposed four weeks previously to airborne infection with the virulent bovine strain of tubercle bacillus, Vallée. Note numerous tubercles of lungs. Spleens slightly larger than normal. (Photograph by Julian Carlile).

bial units per unit volume of nebulized fluid is predictable(3,6). However, without minimizing the importance of the study of the physical aspects of airborne infection, it seems fair to state that preoccupation with this phase of the problem accounted for the complexity and expense of previously described continuous ventilation types of apparatus, thus limiting application of this mode of experimental infection to very few laboratories. The model described here should permit wider use of airborne infection methods with mice as the experimental animals.

Tubercle bacilli, cultivated in Tween-albumin liquid medium, can be used to prepare well dispersed and stable suspensions consisting predominantly of single bacilli, and the numbers of living bacterial cells in such suspensions can be accurately predicted(8). Thus, it is possible with this apparatus and with suspensions of tubercle bacilli prepared in this fashion to induce simultaneously a uniform type of pulmonary tuberculosis in large numbers of mice. It is of some interest that the extent of pulmonary disease induced by this method is much greater than that caused by the same number of tubercle bacilli injected into mice of the same strain and age by the intravenous route(10).

Summary. A safe and relatively inexpensive laboratory apparatus for the experimental airborne infection of as many as 100 mice at a time has been described. It has been used for the production of murine pulmonary tuber-

culosis with tubercle bacilli cultivated in Tween-albumin liquid medium.

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Received April 25, 1952. P.S.E.B.M., 1952, v80.

Effect of Filter Paper, *para*-hydroxybenzoic Acid, and Fixed Tissue on Phagocytosis. (19539)

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Wood *et al.* have described "surface phagocytosis" at length under the following conditions(1-4): "The material with surface to be tested was spread flat in the bottom of a Petri dish lined with filter paper previously soaked in Locke's solution. The leukocyte-pneumococcus mixture was distributed over the test surface, and the dish was sealed and placed in the incubator (37°C) for 1 to 3 hours. In this way the preparations were kept moist. When the Petri dishes were opened, impression smears were made from the test surfaces and were stained with methylene blue." Substances such as glass, paraffin, and cellophane were reported as inactive, while a variety of materials including fixed tissue sections, fiber glass, and filter paper alone promoted phagocytosis. The common denominator in the cited experiments was wet filter paper, which might have been responsible for phagocytosis. The inactive materials were those impermeable to liquid.

This report deals with the influence of filter paper on phagocytosis of *E. coli*. During this work, an active principle in filter paper, *para*-hydroxybenzoic acid, was reported by Davis(5,6) as a new bacterial vitamin and antagonist for *para*-aminobenzoic acid. The effects of *para*-hydroxybenzoic acid and of fixed tissues were also examined.

Methods. E. coli suspensions. Cultures were grown for 18-20 hours in tryptose broth containing 1% dextrose, 3.7 μg Fe/ml, and 0.1 μg thiamin HCl/ml. The bacteria were harvested and washed in saline, standardized turbidimetrically to 4 x 10^{10} cells/ml, and checked by dilution plate counts. The bacteria were killed by treatment with 1% CH_2O for 24 hours at room temperature, washed 3 times in saline, and sterility was checked by plating. The number of bacteria employed in each set of comparative experiments was the same but varied among the different sets.

Filter paper. Strips measuring 1 x 4 or 1 x 5 cm of Whatman No. 1 or No. 42 paper were rolled and placed in the bottom of 12 ml tapered centrifuge tubes. Bacterial suspensions containing from 4 x 10^9 to 2.4 x 10^{10} organisms in 0.4 to 0.6 ml were pipetted into these tubes and allowed to remain in contact for 30 to 60 minutes, after which the filter paper was squeezed out and removed. Chamber counts in several instances showed no significant loss of bacteria after this treatment. *Para-hydroxybenzoic acid.* Eastman C.P. *para*-hydroxybenzoic acid, either free or as the Na-salt, was used in H_2O solutions containing from 0.00001 to 1 mg/ml. One to 3 ml of these concentrations of *para*-hydroxybenzoic acid were added to 2.5 x 10^9 to 6 x 10^9 cells for 30 to 60 minutes, centrifuged, and the cells resuspended in saline with or without extra washing in saline or Krebs-gelatin. *Tissue.* Washed frozen sections 5-25 μ thick of for-

TABLE I. Effect of Different Materials on Phagocytosis of *E. coli.*

Test substance	Observations	Percentage neutrophils containing bacteria—Mean ± S.D.	Diff. ± P.E. diff.	Diff. / P.E. diff.
A. Saline	36	36.6 ± 17.7		
B. Filter paper	32	55.4 ± 22.4	B–A 18.8 ± 3.3	B–A 5.7
C. *para*-hydroxybenzoic acid	40	57 ± 18.7	C–A 20.4 ± 2.8	C–A 7.3
D. Tissue	34	21.9 ± 18.9	D–A 14.7 ± 3	D–A 4.9

malin-fixed liver, kidney, lung, spleen, brain, heart, or lymph node were used individually or in combinations, in the same fashion as filter paper.

Controls. Bacterial suspensions were treated with saline, and run concurrently with filter paper, *para*-hydroxybenzoic acid, and tissue.

Phagocytosis. This was measured quantitatively by a previously described method(7), using dog blood cells washed 6 times in Krebs-gelatin solution. The washed dog blood cells were mixed with an equal volume of Krebs-gelatin solution; 0.1 ml of this suspension was added to 0.1 ml of the bacterial suspensions cited above in an 8 mm test tube and mixed in a rotator(8) revolving 24 or 36 RPM for 30 minutes at 37°C. The number of bacteria in each set of comparative experiments ranged from 1×10^9 to 4×10^9. Smears were prepared and stained with Giemsa-Jenner. The percentage of 50 neutrophils containing bacteria employed in individual tests is indicated in the text.

Results. Under standardized conditions for measuring phagocytosis(7) with washed dog blood cells, 36.6 ± 2% of neutrophils contained *E. coli;* with previous contact with filter paper the mean percentage was 55.4 ± 2.7; with *para*-hydroxybenzoic acid 57.0 ± 2.0; and with tissue sections 21.9 ± 2.2. Thus filter paper and *para*-hydroxybenzoic acid enhanced, and tissue inhibited, phagocytosis (Table I).

The stimulatory effect of *para*-hydroxybenzoic acid was related to the concentration between 0.001 and 1 mg per ml (Table II), and could be plotted as a straight line semilogarithmic ratio.

Filter paper and *para*-hydroxybenzoic acid affected the bacteria directly; no opportunity was afforded for their action on the leukocytes in these experiments. This effect with extremely small amounts was not removed by washing and therefore resembled opsonin action. Since the bacteria were dead during exposure to these "opsonizing" agents, there was no production of opsonizing materials during growth, observed in other unpublished studies.

TABLE II. Effect of *para*-hydroxybenzoic Acid on Phagocytosis of *E. coli.*

No. of *E. coli*	*para*-hydroxybenzoic acid—mg/ml .0	.001	.01	.1	1
	% neutrophils containing *E. coli*				
6×10^9	32	50	56	56	72
3×10^9	16	40	48	52	68

The effects ascribed to "surface" phenomenon in phagocytosis may have been due to wet filter paper. Surfaces favorable to phagocytosis were freely permeable to liquid, and those unfavorable were impermeable. In the light of these data and Wood's experiments, the claim that a favorable "surface" enhances phagocytosis is open to question.

Conclusions. 1. Phagocytosis of formalin-killed *E. coli* was promoted by previous contact with filter paper or with *para*-hydroxybenzoic acid. The latter, an active principle in filter paper, was effective in concentrations of 1 to 0.001 mg per ml. Fixed tissue inhibited phagocytosis. 2. The activity of the several surfaces reported by Wood *et al.* to promote phagocytosis may be ascribed to the wet filter paper in their experiments. These observations open to question the phenomenon of "surface" phagocytosis.

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Received April 29, 1952. P.S.E.B.M., 1952, v80.

Protection of Mice Against X-Irradiation by Spleen Homogenates Administered After Exposure.*† (19540)

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Protection of animals against the lethal or deleterious effects of ionizing radiations is now possible by means of several chemical substances administered prior to, or during X-irradiation: for example, cysteine(1), glutathione(2), or sodium nitrite(3). In addition, anoxic anoxia during irradiation is also effective(4). None of these measures, however, has been shown to be protective when carried out *after* irradiation. Recent studies by Jacobson *et al.*(5) suggest the presence of a spleen factor which appears to be effective *after* irradiation exposure. These workers transplanted 4 spleens from young non-irradiated mice (1-12 days old) into the peritoneal cavity of adult CF-1 mice immediately after exposure to 1025 r whole-body X-irradiation. Under these conditions, survival of the irradiated mice was increased, and regeneration of hematopoietic tissue was hastened.

Several initial experiments in this laboratory in which spleens from young mice were implanted into irradiated mice (650 r) confirmed the protective effect of this procedure in LAf_1 mice. The implants became vascularized, grew, and were still present and intact 6 weeks after implantation. As a first approach to isolating and ascertaining the nature of the splenic factor, studies were performed using spleen homogenates.

The preliminary data, described herein, indicate that mice receiving otherwise lethal doses of whole-body X-irradiation are protected by a single intraperitoneal injection of spleen homogenate administered either one hour or as long as 45 hours after radiation exposure.

Materials and methods. LAf_1 mice of both sexes, approximately 7 to 11 weeks old and weighing 20 to 26 g, were used in the irradiation studies. The animals were allowed free access to food (Purina Laboratory Chow) and to tap water. In all experiments the control and experimental animals were matched with respect to sex, age, and body weight; they were irradiated simultaneously and caged together (8 or 10 per cage). A Westinghouse Therapy Unit was used as the radiation source. The radiation factors were: 250 KVP; 15 ma; filter, 0.5 mm Cu plus 1 mm Al; HVL, 1.5 mm Cu; skin to target distance, 100 cm; dosage rate, 25 r per minute, as measured with a Victoreen r-meter placed in air at the position of the mice. Each radiation dose was delivered in a single exposure. During irradiation the mice were contained in individual, perforated lusteroid centrifuge tubes placed radially on a circular wooden turntable platform which rotated at 3.5 r.p.m. to obtain uniformity of radiation dosage.

In a typical experiment, 20 to 30 young LAf_1 mice (1 to 3 weeks old) were sacrificed by severing the cervical spine. The spleens were removed immediately and suspended in

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official, or reflecting the views of the Navy Department.

† This work was supported, in part, by funds provided by the Bureau of Medicine and Surgery, U. S. Navy Department.

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TABLE I. Protection of Mice by a Single Intraperitoneal Injection of Spleen Homogenate Administered after Whole Body X-irradiation.

Exp.	No. of mice*	Age (wk)	X-ray dosage (r)	Treatment†	No. of spleens	Survival at 30 days No.	Survival at 30 days % survival
I	9	7	650	Spleen homogenate	.9	9	100
	10	7		Phosphate buffer		0	0
II	14	8	650	Spleen homogenate	2	11‡	79
	19	8		Phosphate buffer		4	21
III	13	9	650	Spleen homogenate	1.7§	11	85
	13	9		Phosphate buffer		1	8
IV	10	10	700	Adult spleen homogenate	1.6	8	80
	10	10		Phosphate buffer		4	40
V	15	9	700	Spleen homogenate	1.3	14	93
	10	9		Phosphate buffer		1	10
VI	15‖	10–11	800	Spleen homogenate	.9	8	53
	14‖	10–11	800	" "	1.8	11	79
	11‖	10–11	800	Phosphate buffer		0	0

* Female mice were used in all experiments except as otherwise noted.
† Spleen homogenates were inj. within one hr after irradiation except as otherwise noted.
‡ Three animals died on 4th day post-irradiation.
§ Injected 45 hr post-irradiation.
‖ Male mice.

3 ml of cold M/15 phosphate buffer (pH 7.2). They were then homogenized in a motor-driven ground-glass Ten Broeck tissue homogenizer which was immersed in an ice-water bath. The homogenate was diluted with phosphate buffer to a volume suitable for injection, usually equivalent to 20 spleens per 10 ml of homogenate. Within 15 minutes after preparation the homogenates were injected into irradiated adult mice by the intraperitoneal route using a 26-gauge needle. Between 0.5 and 1.0 ml of homogenate was injected into each animal. Control irradiated mice were injected with equivalent volumes of phosphate buffer. The homogenate was injected within one hour after irradiation in all experiments except one, in which it was injected 45 hours after exposure. The mice were observed daily for mortality and gross signs, and were weighed at 24- or 48-hour intervals over the 30-day period following irradiation. Survival up to 30 days post-irradiation, and average body weight changes were used as criteria for evaluation of protection elicited by the spleen homogenates.

Microscopic examination of the homogenate revealed the presence of apparently intact cells, in addition to cell fragments and cell nuclei. Preliminary estimates indicate that 60 to 70% of the spleen cells were disrupted by the homogenization procedure.

Results. The survival data obtained in 6 separate experiments with spleen homogenates (a total of approximately 160 mice) are summarized in Table I. The radiation doses used in these experiments (650, 700 and 800 r whole-body X-irradiation) include the lethal and supra-lethal§ range for mice. The data reveal that a single parenteral spleen homogenate injection into adult X-irradiated mice within one hour after radiation exposure confers considerable protection in these mice. Each mouse received the equivalent of 1 to 2 spleens from young non-irradiated mice.

In Exp. I, 100% of the spleen-homogenate-treated animals survived a 650 r dosage of X-irradiation, whereas none of the control mice survived. In Exp. II, using a larger group of mice, only 4 out of 19 control animals (21%) survived a 650 r dose, and 11 out of 14 (79%) of the spleen-homogenate-treated

§ Defined here as that range of doses in excess of the minimum dose required to kill 100% of animals in 30 days.

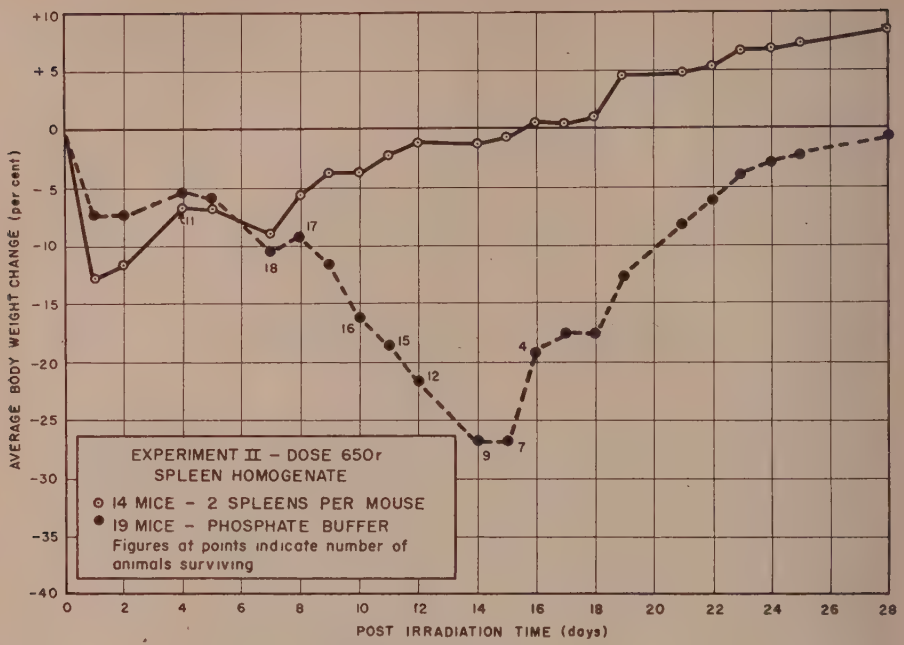

FIG. 1. Body weight changes in female mice injected with spleen homogenate one hour after 650 r x-irradiation.

mice survived.‖ Body weight changes, expressed as percent of initial body weight in Fig. 1, also illustrate the protective effect of the spleen homogenates.

In Exp. III, in which spleen homogenate was injected into irradiated mice 45 hours after 650 r X-irradiation, protection was also obtained. The survival data in Table I, as well as the body weight changes plotted in Fig. 2, indicate that the spleen homogenate is protective even when given approximately 2 days after irradiation. The efficacy of spleen homogenate protection was also demonstrated in mice subjected to 700 and 800 r whole-body exposure. Of the control group of 10 mice exposed to 700 r in Exp. V, one was alive at 30 days, and 14 out of 15 (93%) of the spleen-homogenate-injected group survived.

The possibility that the protective spleen factor is present, at least to some extent, in adult spleen tissue as well as in young spleens is suggested by the results of Exp. IV. The equivalent of 1.6 spleens from adult non-irradiated mice (5 weeks old) was injected into each of 10 mice within one hour after they had received 700 r. Twenty percent of this group was dead at the conclusion of the 30-day observation period, whereas 60% of the phosphate-buffer-injected group was dead at 30 days. The weight curves for these animals in Fig. 3 likewise illustrate this protection by homogenates of spleens from adult mice. Thus the weight loss of the control irradiated mice was 20% on the 12th day, compared with a weight loss of only 13% for the spleen-homogenate-injected group on the 12th day.

Finally, the effect of spleen homogenate from young mice on mice subjected to 800 r irradiation was investigated in Exp. VI. Of 14 mice receiving homogenate equivalent to 1.8 spleens, 11 were alive at 30 days (78%); whereas of 15 mice receiving homogenate equivalent to 0.9 spleens, 8 survived 30 days (53%). None of the control animals was

‖ Survival time of the 3 mice that died in this experiment was 4 days, as opposed to approximately 11 days as uniformly observed in this dose range at this Laboratory. It seems likely, therefore, that mechanisms other than radiation, *per se*, may have accounted for these deaths.

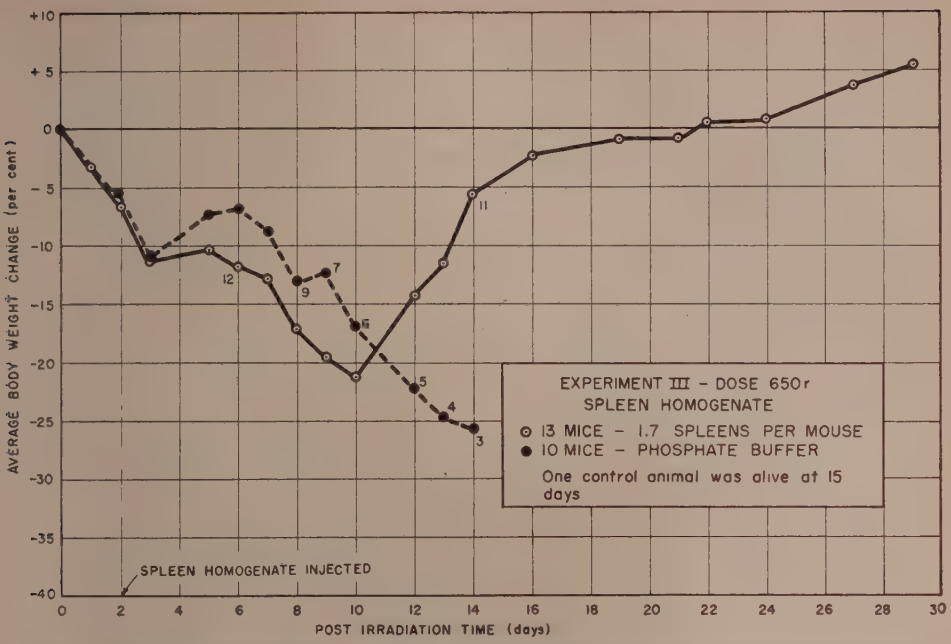

FIG. 2. Body weight changes in female mice injected with spleen homogenate 46 hours after 650 r x-irradiation.

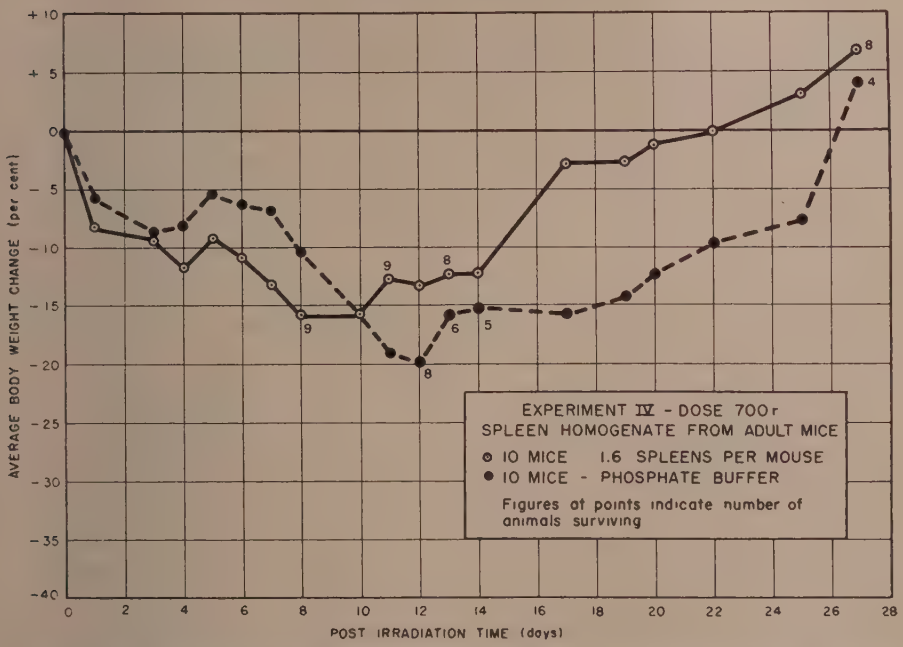

FIG. 3. Body weight changes in female mice injected with adult spleen homogenate one hour after 700 r x-irradiation.

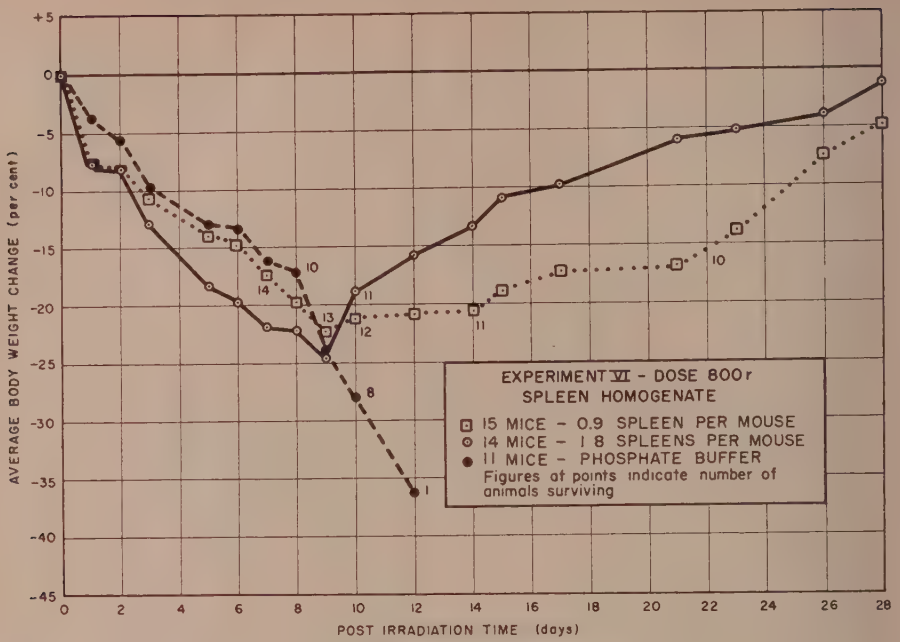

FIG. 4. Body weight changes in male mice injected with spleen homogenate one hour after 800 r x-irradiation.

alive at the conclusion of the 30-day observation period. Fig. 4 presents the post-irradiation body weight changes in these animals. The data suggest a possible quantitative relationship between the amount of spleen substance injected and the degree of protection against X-irradiation obtained; *i.e.*, 1.8 spleens elicited a greater degree of protection than did 0.9 spleens.

Discussion. The results appear to warrant a positive conclusion as to the presence of a factor in homogenates of spleens from young mice which greatly modifies the response of irradiated adult mice when injected parenterally after irradiation. This modification in response is reflected—as the present data demonstrate—in increased survival, minimal body weight losses and in earlier body weight recovery in spleen-homogenate-treated mice, as compared with control irradiated animals. The protection afforded by spleen homogenate injection is not attributable to a non-specific tissue effect, since spleen homogenates lose their protective activity after being subjected to ultra sound in a crystal Ultra-Sonorator.

The present positive finding of protective activity in *adult* spleen homogenate apparently is contrary to the negative findings of Lorenz *et al.*(6). These investigators reported the failure of post-irradiation adult mouse spleen brei injections (intravenous and intraperitoneal) to protect LAf_1 mice from 900 r whole-body X-irradiation. Each mouse received approximately 50 to 100 mg of spleen brei. In the present studies, it will be noted, the equivalent of 1.6 adult spleens (ca 150 mg) was required to protect mice which had received 700 r (Exp. IV). Thus in the Lorenz experiments both the higher radiation dosage and the smaller amounts of spleen substance administered may have been responsible for their negative findings.

Examination of the post-irradiation body weight data in the spleen-homogenate-treated mice reveals a turning point in their weight loss trend which occurred consistently at approximately 8 days after injection of spleen homogenate. It is at this time that a divergence in the average body weight losses between the spleen-homogenate-treated mice and

the corresponding control group of animals is noted. This finding is in agreement with that reported by Jacobson *et al.* for spleen-shielded mice exposed to 1025 r(7). By histological technics these workers have shown that bone marrow recovery appears to be complete in mice by the 8th day post-irradiation. Since, however, first evidence of a reversal of peripheral leucocyte and reticulocyte depression apparently occurs on the 6th day following irradiation(7), it would appear that body weight recovery of the spleen-homogenate-treated mice in the present investigation occurs only after the appearance of bone marrow recovery.

Multiple injections of spleen homogenates were not employed in the present investigation. It seems reasonable to suppose, however, that such treatment might confer greater protection upon irradiated mice than do single injections, especially at higher X-ray dose levels.

The present demonstration of the post-irradiation protection afforded by injectable spleen homogenates in irradiated mice provides a research tool of promise for investigation of the nature of the protective spleen factor. Studies along these lines are now in progress.

Summary and conclusions. 1. The mortality of adult LAf_1 mice, exposed to whole-body X-irradiation in doses of 650, 700 and 800 r, was either prevented or reduced markedly following a single intraperitoneal injection of spleen homogenate administered either one hour or as long as 45 hours after radiation exposure. The equivalent of 1 to 2 spleens was injected into each irradiated mouse. The protective factor was present in homogenates from spleens of young mice (1 to 3 weeks old), and to a smaller degree in homogenates of spleens from adult mice. 2. The post-irradiation protection afforded by spleen homogenate injection was reflected in minimized weight losses observed in the spleen-homogenate-treated mice as compared with the control animals. The data suggest a possible quantitative relationship between the amount of spleen substance injected and the degree of protection obtained.

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Received April 4, 1952. P.S.E.B.M., 1952, v80.

Human Assay of Three New Mercurial Diuretic Agents: A Promising Preparation for Oral Use.* (19541)

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A method for the bioassay of diuretic agents in ambulant patients with congestive failure

* This investigation was supported (in part) by a research grant from the National Heart Institute of the National Institutes of Health, U. S. P. H. S., and by the Diuretic Fund of Department of Pharmacology, Cornell University Medical College.

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was developed in our clinics(1). This method was employed in the present study. The plan provides for the comparison of the Unknown with the Solution of Mercuhydrin Sodium (meralluride sodium) by intramuscular injection, which we have adopted as the "Standard." The measure of response is the loss of body weight 24 hours after the dose. From dose-response curves for the Unknown and the Standard, the potency of the Unknown is determined. The assay is so designed as to supply data suitable for statistical analysis, and the precision of the difference in potency between the Unknown and the Standard is expressed in terms of confidence limits. This method has provided an efficient tool for the screening of new diuretic agents and for the investigation of their relative efficacy by various routes of administration.

In the present study, this method was used in the screening of 3 organic mercurial compounds which displayed diuretic action in laboratory animals(2-4). In addition to the diuretic activity, observations for possible toxicity were made: gastrointestinal effects; local irritant action; a complete blood count before the first dose and within 2 weeks after the last dose; similarly for blood urea nitrogen; urine examined during the action of each of the large doses, as well as before and within 2 weeks after the study. The results form the subject of this report.

Preparations. There were 4 preparations. The Standard was the injectable solution of Mercuhydrin sodium of commerce as supplied in sterile multiple-dose vials of 10 cc each. Of this solution, each cc is stated(5) to contain 123 mg of meralluride sodium and 9.4 mg of free theophylline. Two of the new agents were thiol compounds. Preparation "E" (3-carboxymethylmercaptomercuri-2-methoxy propylurea) was supplied in tablets containing 20 mg for oral administration. Preparation "F" (3(α-carboxyethylmercaptomercuri)-2-methoxy propylurea) was supplied in a sterile vial containing 439 mg of powder which we dissolved in 10 cc of water. This was used for intramuscular injections which were made within the hour. Of this solution, each cc represented 43.9 mg of the compound. This material was also supplied in tablets for oral administration, each containing 21.75 mg. Preparation "D" (3-chloromercuri-2-methoxy propylurea) represents meralluride from which the succinyl group was removed from one end and the theophylline from the other. It was supplied for intramuscular injection in the form of sterile ampules containing 2 cc of solution, each cc of which is stated to contain 23.85 mg of the compound. It was also supplied in the form of tablets for oral administration, each tablet stated to contain 18.35 mg of the drug. The tablets were always given at one time, in single doses of from 1 to 9 tablets. The comparisons as shown in the charts were made in terms of molecular weight, but since a molecule of each compound contains 1 atom of mercury, the values for relative potency as expressed in millimols of the compound or in milligrams of mercury are identical. It may be mentioned parenthetically that the significance of the common expression of relative potency of organic mercurials in terms of mercury content is open to question.

Results. None of the compounds in these assays produced changes in the blood count, blood urea nitrogen, or urine. They all caused gastrointestinal symptoms by oral administration and the incidence of these in relation to dosage and diuretic effectiveness is described below. When the interpretation of the weight loss was complicated by vomiting or watery diarrhea, the values in such cases were excluded from the calculation of potency.

An attempt to assay preparation "E" proved unsuccessful. Thirty-three patients received a total of 67 oral doses ranging from 20 to 100 mg. In the group of 21 patients who received the 60 mg dose, 48% developed in a period of hours, one or more of the following gastrointestinal symptoms: nausea, abdominal cramps, diarrhea, and in one case bloody stools. The diuretic effect was negligible, average weight loss of 0.12 lb 24 hours after the dose. With the 100 mg dose in 11 patients, 64% developed the foregoing symptoms, in one instance also vomiting. Again, the diuretic response was negligible, average weight loss of 0.18 lb in 24 hours. The possibility of delayed diuretic response was examined in a group of 10 patients (12 doses)

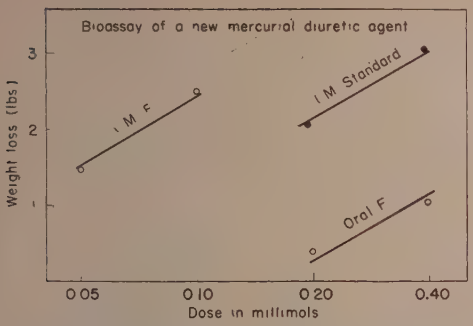

FIG. 1. Comparison of 3(α-carboxyethylmercaptomercuri)-2-methoxy propylurea by oral and intramuscular administration with the Standard, intramuscular mercuhydrin.

who were weighed 24 hours and again 48 hours after doses of 60, 80, and 100 mg. No delayed action was in evidence; their average weight loss in 24 hours was 0.35 lb, and in 48 hours the average weight returned to a level above that before the dose. "E" was manifestly unsuitable as an oral diuretic.

Fig. 1 shows the potency of preparation "F" by the intramuscular and by the oral route, determined in one assay. The 3 curves represent the same 37 patients, and each is based on 74 doses. The intramuscular doses were 0.5 cc (21.95 mg) and 1.0 cc (43.9 mg). The doses of the Standard (meralluride by intramuscular injection) were 1 cc (123 mg) and 2 cc (246 mg). It may be seen that by the intramuscular route, "F" has 2.5 times the diuretic potency of the Standard in terms of millimols and approximately 3.5 times in terms of milligrams of the compounds.

In the case of "F" by the oral route, patients received single doses ranging from 21.75 to 174 mg. Fig. 1 shows that "F" by the oral route has 9.2% of its potency by the intramuscular route, or conversely it takes approximately 11 times as much "F" by the oral as by the intramuscular route to produce the same diuretic effect. If it may be assumed, as has been shown in the case of other mercurial agents(6,7) that by the intramuscular route absorption is nearly complete, there is indication that only about 9% of a dose is absorbed from the gastrointestinal tract (Table I). The largest oral doses, 174 mg, which gave rise to gastrointestinal symptoms of the type listed above after one-fourth of the number of such doses given, yielded a small diuretic effect equivalent to that resulting from less than 0.5 cc (61.5 mg) of the Standard (meralluride by intramuscular injection). As in the case of preparation "E", there was no evidence of delayed diuretic response; 21 patients who received the 174 mg dose showed an average weight loss of 0.8 lb in 24 hours and in 48 hours a return to an average of 0.45 lb below the control weight. Preparation "F" did not appear to be especially promising as a diuretic agent for oral administration.

Fig. 2 shows the results of a preliminary assay of preparation "D" by oral administration. There were 3 dose levels, 73.4, 110.1, and 165.15 mg. The oral administration shows a potency of 70.7% of the Standard in terms of millimols and 122% in terms of milligrams of the compounds. At the level of dosage, 110.1 mg (6 tablets), a fairly good diuretic effect was obtained, equivalent to the effect of 1.1 cc of mercuhydrin in this group of patients. This dose produced gastrointestinal symptoms after 30% of the number of such doses given. These results may be compared to those of preparation "F" (see above) with which a dose causing an approximately similar incidence of local toxicity produced a diuretic effect half as much. As in the case of the other two compounds, preparation "D" also failed to show any diuretic action de-

TABLE I. Potency and Precision of the Assays.*

Route	Potency† expressed as % of: Mercuhydrin i.m. as standard	Particular unknown i.m. as standard	Confidence limits at P = .05
3 (α-carboxyethylmercaptomercuri)-2-methoxy propylurea			
Intramusc.	252.2		142.9 & 364.4
Oral	23.3		7.1 " 39.8
"		9.2	3.8 " 14.3
3-chloromercuri-2-methoxy propylurea			
Intramusc.	249.7		187.5 " 321
Oral‡	71.0		57.6 " 87.4
"		28.5	21.6 " 36.7

* Calculations according to C. I. Bliss, Statistical Methods in Vitamin Research, Academic Press, New York, 1951.

† Molecular terms.

‡ Combination of two assays.

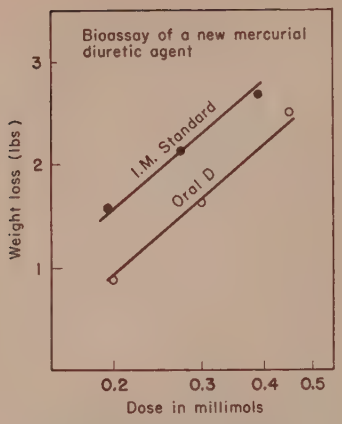

FIG. 2. Comparison of 3-chloromercuri-2-methoxy propylurea by oral administration with the Standard, intramuscular mercuhydrin.

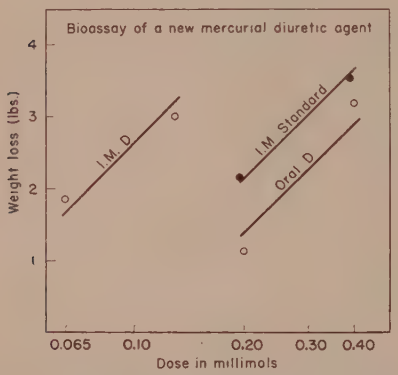

FIG. 3. Comparison of 3-chloromercuri-2-methoxy propylurea by oral and intramuscular administration with the Standard, intramuscular mercuhydrin.

veloping after the 24-hour period; 23 patients who received 33 doses of from 55.05 to 165.15 mg by the oral route showed an average weight loss of 2.2 lb at the 24-hour weighing and an average gain of 0.3 lb above this at the 48-hour weighing. Preparation "D" clearly presented more favorable properties than either "E" or "F". Since the assay shown in Fig. 2 was made with only 15 patients, a more definitive assay was carried out. The results are shown in Fig. 3. The 2-dose method was used. The same 27 patients are represented in each of the three curves, and each is based on 54 doses. The potency of preparation "D" by the oral route in this larger assay confirms the value established in the preliminary assay, 71.2% of that of the Standard (by intramuscular injection). Preparation "D" by the oral route has 28.5% its potency by the intramuscular route, and again, on the basis of fairly complete absorption from the intramuscular injection, this value would indicate the extent of its absorption from the gastrointestinal tract.

Preparation "D" is a much more potent material than meralluride as shown in the comparison in which both were given by intramuscular injection. The curve represents "D" in doses of 23.85 mg and 47.7 mg respectively. It may be seen that preparation "D" has 2.5 times the potency of the Standard in terms of millimols and 4.3 times in terms of milligrams of the compounds (Table I).

It may be mentioned that, while the first two preparations which were thiol compounds were relatively painless by intramuscular injection, preparation "D" caused considerable local discomfort and pain in the muscle area of injection.

The data on gastrointestinal symptoms produced by preparation "D" have been assembled in Table II. The general pattern of the response of patients to the local irritant action in the gastrointestinal tract was substantially similar for all three compounds. There are marked variations in susceptibility to the irritant action in the gastrointestinal tract in the same patient at different times, and in different patients. A small dose sometimes produced these unpleasant symptoms in a particular patient who at other times tolerated well more than 4 times as much. As may be seen in Table II, about one-half of the patients tolerated without gastrointestinal symptoms a dose which was more than 4 times as large as the one which in others produced gastrointestinal irritation. Nevertheless, there is a general dosage-response trend with respect to this effect: When expressed in terms of patients, the incidence of gastrointestinal reactions rose from 18 to 64% as the dose was gradually increased through a 4-fold range; when expressed in terms of doses, an essentially similar trend was in evidence.

Comment and Conclusions. Three organic mercurial preparations possessing diuretic ac-

TABLE II. Gastrointestinal Irritation after Oral Administration of 3-chloromercuri-2-methoxy propylurea.

	Doses (mg)					
	36.7	55.05	73.4	110.1	146.8	165.15
No. of patients who received dose	14	3	38	15	32	16
% of patients who developed symptoms*	18	100	25	37	64	53
No. of doses administered	15	3	55	20	36	21
Types of gastrointestinal toxicity						
Nausea	1		3		5	
Abdominal cramps		1	3	2	3	4
Vomiting	2	1	1		9	1
Diarrhea	1	1	4	4	5	5
% of doses causing gastrointestinal toxicity	27	100	20	30	61	48

* Where a patient received a particular dose twice, but developed gastrointestinal symptoms after only one of them, it was counted as ½.

tivity were assayed by the oral route against the Standard, mercuhydrin solution given intramuscularly, in patients with congestive failure. Of the three materials, the 3-chloromercuri-2-methoxy propylurea proved to be the most effective, producing with oral doses a diuretic response equivalent to results obtained by the conventional doses of intramuscular mercuhydrin. The diuretic potency of this compound when given orally is somewhat more than one-fourth of its potency by intramuscular injection, and by the latter route 4.3 times (in milligrams) as potent as intramuscular mercuhydrin. We are not aware of any mercurial diuretic with such a favorable ratio of intramuscular to oral potency, namely 4:1. In the case of the thiol compound we tested in this study, the ratio was 11.1. In another study(7) in which mercuhydrin was tested by the intramuscular and oral routes, this ratio was 24:1. There still remains the problem of gastrointestinal irritation. By the method employed in the bioassay, it was necessary to give the total dose at one time, in the case of the larger doses as many as 9 tablets. This, therefore, subjected the local irritant action to a rigorous test. As the results stand, it appears that approximately one-half of the population with congestive heart failure might be able to tolerate by the oral route doses of this compound which produce highly effective diuretic responses. This is as far as the investigation in clinical pharmacology has carried the problem. It is now necessary to establish the most satisfactory dosage plans for the use of this material by the oral route. If approximately one-half of the population can tolerate as many as 9 tablets given at one time without gastrointestinal distress, it may well turn out that by dividing this amount into several fractions taken at intervals during the day, satisfactory diuretic effects may be obtained with less interference from gastrointestinal symptoms. The protracted use of the material over periods of weeks and months may disclose other problems which are not revealed by the single dose bioassay method. It remains for clinical trials to decide these matters.

The authors are indebted to Mrs. Rita Simpson and Miss Susan Otto for technical assistance.

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Received April 29. 1952. P.S.E.B.M., 1952, v80.

Anaphylactic Shock in the Pertussis Vaccinated Mouse. (19542)

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The sensitivity of the white mouse to histamine has been referred to as being 1/1000 that of the guinea pig(1). Recently, however, Parfentjev and Goodline(2) reported that the sensitivity to histamine could be increased about 50-fold by a preliminary inoculation with *Hemophilus pertussis* vaccine. This finding has been elaborated and enlarged in subsequent publications(3-9). Similarly, the white mouse is known to be markedly resistant to anaphylactic sensitization and shock. This observation correlated to their refractoriness to histamine has led to the belief(10) that histamine plays no direct or significant role in the mouse as it does in other species(11). In restudying the relationship between histamine and anaphylactic shock in the white mouse a procedure was evolved by which sensitization and fatal shock could be produced uniformly. This technic is the basis of the following preliminary report.

Methods and results. Female, white mice* were inoculated intraperitoneally with 0.5 ml of a mixture containing about 8,750 million phase I organisms of *H. pertussis* vaccine, Lederle, and 0.03 ml horse serum. On the fourth subsequent day a series of animals tested with 0.5 ml of a histamine diphosphate solution in saline given intraperitoneally showed them to be markedly sensitive. Whereas the LD_{50} of histamine diphosphate for a control series of unvaccinated animals was 38 mg for a 20 g mouse, the LD_{50} for the vaccinated group was 0.8 mg. The mortality rate was determined as the number of animals dead within 24 hours/number of animals injected.

Fifteen days after sensitization the vaccinated mice were challenged by the intravenous administration of 0.1 ml of horse serum. The mortality rate of this series was 95/102 as compared to a rate of 3/66 in a control series of animals, sensitized to horse serum as above but not receiving the *H. pertussis* vaccine. With respect to histamine sensitivity after 15 days, the vaccinated mice still showed a mortality of 4/4 when injected with 1 mg and 1/4 with 0.5 mg of histamine diphosphate in solution.

Of several vaccines tested in an identical manner for their enhancement of anaphylactic sensitivity, only *Brucella abortus* vaccine, Sharp and Dohme, 1200 million organisms per mouse, produced any significant increase in mortality rate under comparable conditions, 7/16.

Summary. 1. It has been confirmed that *H. pertussis* vaccination of white mice produces a 50-fold increase in histamine sensitivity. 2. Mice immunized by an intraperitoneal injection of a mixture of *H. pertussis* vaccine and horse serum become markedly anaphylactogenic to a challenging dose of the serum. *Brucella abortus* vaccine has a somewhat similar effect on anaphylaxis.

* The female, white mouse has been found to be more sensitive than the male to histamine both before and after vaccination(12).

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Received May 5, 1952. P.S.E.B.M., 1952, v80.

Cortisone and Roentgen Radiation in Combination as Synergistic Agents for Production of Lethal Infections.* (19543)

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This paper reports a highly efficacious method for the production of rapidly progressive lethal infections. Cortisone and x-ray when employed in combination for the preparation of test animals alter their resistance to enhance remarkably the pathogenicity of a variety of infectious microbiological agents. To date studies in this laboratory have applied successfully this method for the enhancement of the pathogenic effects of poliomyelitis virus, type 2, Lansing strain(1); Coxsackie virus, subgroup A, type 4, Minnesota strain(2); *Blastomyces dermatitidis,* yeast phase(3); *Candida albicans*(4); *Streptococcus pyogenes hemolyticus,* group A, type 28(5); and *Mycobacterium tuberculosis,* var. hominis(5). We record this supplementary note at the present time to permit without delay the application of the method to laboratory studies of exotic agents and infectious agents of limited host receptivity. The ready applicability of the method is illustrated in this report by the results that were obtained in 4 experiments.

Cortisone and x-ray, when employed singly, have been reported to exert an enhancive effect upon the severity of infection when it is induced by any of a variety of infectious agents. Measurable alterative effects of cortisone to the disadvantage of the host are recorded for a wide range of viruses, bacteria, and a single fungus. The viruses include influenza A(6), influenza B(6), mumps(6), poliomyelitis, type 2, strain MEF-1(7), pneumonia virus of mice(8); Coxsackie virus, subgroup B(9); vaccinia(10), West Nile(11), Ilheus(11) and Bunyamwera(11). The bacteria studied were *Staphylococcus aureus*(10), group A streptococci(12-14), *Mycobacterium tuberculosis* (15) *Diplococcus pneumoniae*(16), *Brucella spp.*(17) and *Treponema pallidum*(18). The fungus was *Trichophyton mentagrophytes.* The variable effects of x-irradiation on infection and immunity were ably reviewed recently by Taliaferro and Taliaferro(19).

Materials and methods. Infectious agents. The microorganisms used were: 1) poliomyelitis virus, type 2, Lansing strain, derived from a sample originally given us by Dr. J. E. Salk; 2) Coxsackie virus, subgroup A, type 4, Minnesota 1 strain which was isolated in this laboratory; 3) *Candida albicans,* the Asbury strain which was kindly supplied by Dr. J. R. McGrath; 4) *Blastomyces dermatitidis* isolated from a patient with blastomycosis. These infectious agents were prepared for injection as follows: 1) The test suspension of Lansing virus employed for intracerebral inoculation, 0.03 ml, was the supernatant fluid prepared by centrifugation at 2000 r.p.m. for 10 minutes of a 10% mouse brain suspension in 0.85% NaCl. 2) The inoculum of the Coxsackie virus, 5 million LD_{50} per 0.1 ml, was the Seitz-EK filtrate of the supernatant fluid derived by centrifugation at 4000 r.p.m. for 20 minutes from a 10% tissue suspension. 3) *Candida albicans* was injected intraperitoneally, 0.5 ml, as cells which had been grown for 48 hours at 30°C on Sabouraud's dextrose agar and made ready for injection by diluting them, 1:250, in 0.85% NaCl. 4) *Blastomyces dermatitidis* was grown on Francis' blood dextrose cystine agar at 37°C for 4-5 days, centrifuged horizontally at 1500 r.p.m. for 5 minutes and resuspended in saline, 1:200 for injection, 1.0 ml, intraperitoneally. *Mice.* Swiss albino mice, CFW strain, were employed in groups of 5 to 25 for test and for titration. The age of the mouse was determined by the

* Aided by grants from the National Foundation for Infantile Paralysis, and the Graduate School Research Fund, University of Minnesota and by a contract with the Office of Naval Research.

† Part of the material will appear in theses in partial fulfillment of the requirements of the degree, Doctor of Philosophy by Mr. Roth and of Master of Science, by Mr. Graham and Mr. Mira.

infectious agent under study: Lansing virus, 21 days; Coxsackie virus, either 1-2 days or 5-6 weeks; *Candida albicans* and *Blastomyces dermatitidis,* 8-12 weeks. *Method of radiation.* X-radiation was administered as a single massive dose to the whole body at a target-skin distance of 60 cm. The Roentgen rays were generated by a current of 15 milliamperes having a 250 kilovolt peak. They were filtered through 0.5 mm of copper and 1.0 mm of aluminum (HVL 1.35 mm Cu). The size of the field was 14.0 cm in diameter. The output, as determined by a Victoreen r-meter, was 41.5 r per minute in air. *Cortisone acetate.* Cortisone acetate, 25 mg per ml, (Cortone, Merck) was employed directly, or by diluting it in saline to provide a concentration of 1.0 mg per 0.1 ml, for injection subcutaneously. *Experimental plan.* The experimental plan was to test the enhancive effect in single doses of cortisone, of x-radiation, or of these two agents in combination upon the pathogenicity of selected microorganisms upon injection within the next 24 hours. Each agent, and combination of agents, was titrated in groups of from 5 to 30 mice for determination of the LD_{50}, as calculated by the method of Reed and Muench (20). The animals were kept under observation daily for 30 days. For control purposes cortisone was tested in mice 5-6 weeks of age in a dosage range of from 0.1 mg to 9 mg; x-radiation in a range of 50 r to 800 r. When evidence for toxicity was obtained for cortisone in a dosage greater than 6 mg, for x-radiation in excess of 450 r, and for cortisone and x-radiation in combination in excess of 3 mg and 400 r, respectively, the test doses of these agents were limited to from 2 to 4 mg for cortisone and kept within a range of 200 to 400 r for x-radiation, whether employed singly or in combination. For mice 8 to 12 weeks of age as employed routinely for fungi, the uninfected control mice in groups of 16 over a period of thirty days showed no ill effects from cortisone, 4 mg, or from x-radiation, 400 r, singly or in combination.

Results. Lansing virus. The results of Exp. 1, as presented in Fig. 1, are representative of the findings that were obtained when the Lansing strain of poliomyelitis virus was employed. The LD_{50} titer (Fig. 1a) that resulted in animals which had been prepared by the administration of cortisone, 2.0 mg, and x-radiation, 200 r, was $10^{5.5}$ as contrasted with an LD of $10^{3.9}$ for the control animals that received virus only. Evidence for enhancement of infection also was obtained for Roentgen radiation to yield an LD_{50} of $10^{4.5}$ and for cortisone to result in an LD_{50} of $10^{5.2}$. A more graphic depiction of these findings in Fig. 1b makes it apparent that 7586 LD_{50} for normal mice is enhanced in pathogenic effect approximately a) 4-fold (31,630 LD_{50}) when tested in mice prepared by x-radiation, b) 20-fold (147,900 LD_{50}) by cortisone and c) 40-fold (316,200 LD_{50}) by the two agents in combination.

Coxsackie virus. Since Coxsackie virus (A4, Minn. 1) is highly infectious upon transfer to newborn mice, evidence for enhancement by the agents under study was sought by employing for test an insusceptible host, the adult mouse. A series of experiments involving 250 mice was set up to measure the enhancive effect of cortisone and x-radiation in combination by assay of the LD_{50} for each of the agents singly and by block titration. Fig. 2 depicts the results of LD_{50} titrations based upon the employment of a constant amount of x-radiation and incremental amounts of cortisone within a dosage range of 0.1-4.0 mg a) at 10 days after infection and b) at 30 days after infection. Conversely, Fig. 3 makes known the results at 10 and 30 days of LD_{50} titrations when the dose of cortisone was kept constant and x-radiation was employed in a dosage range of from 50 r to 400 r. For evaluation of these results and for control purposes, it was established that cortisone or x-radiation singly, or cortisone and virus in combination, was without apparent effect upon the recipient mice when observed at either the 10- or 30-day period. Moreover, it was learned for the higher dosage ranges of either agent and virus that 3.7 mg, or more, of cortisone were required in combination with virus to establish an LD_{50} titer and that 321 r, or more, of x-radiation in combination with virus were required to establish an LD_{50} titer. Fig. 2 and Fig. 3 present findings summarily which show that

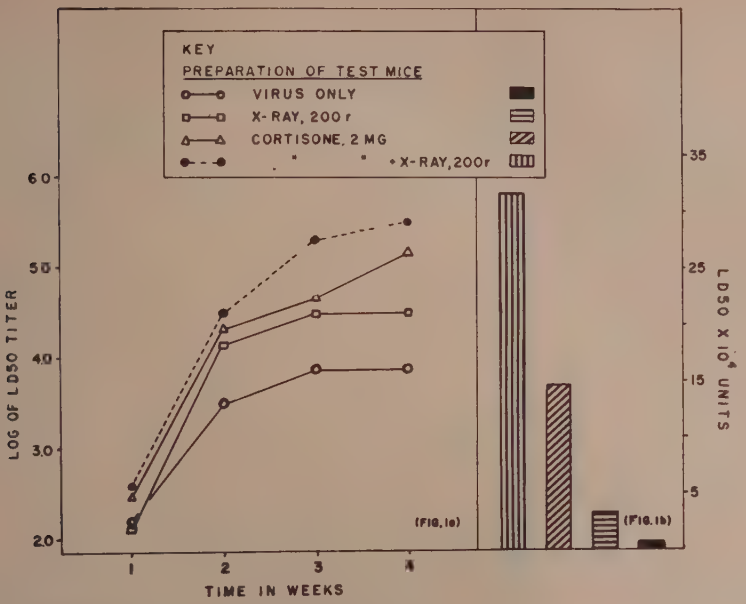

FIG. 1. The enhancive effect of cortisone and x-radiation, singly and in combination, upon the LD_{50} titer of poliomyelitis virus, type 2, Lansing strain.

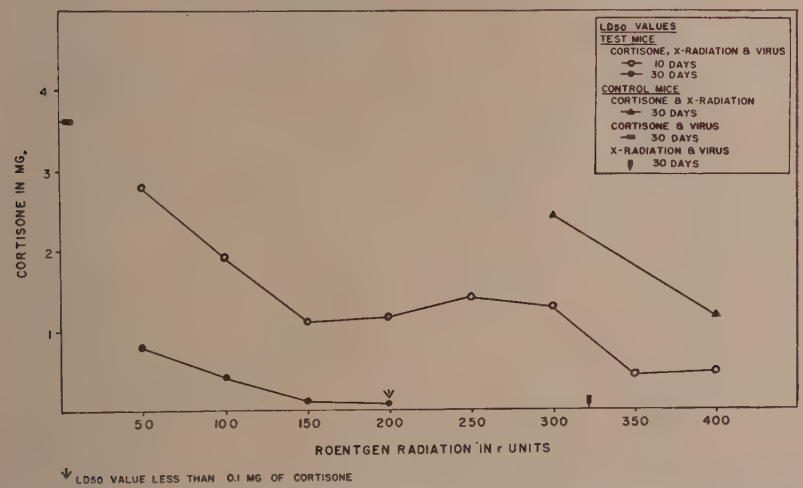

FIG. 2. The synergistic effect of cortisone and roentgen radiation on the pathogenicity of Coxsackie virus in resistant adult CFW mice as indicated by the LD_{50} value for a fixed amount of x-radiation in combination with graded doses of cortisone.

cortisone and x-radiation when employed in combination for preparative treatment of an insusceptible host, the adult mouse, operated synergistically over a wide range for each agent to render Coxsackie virus lethal. The synergistic enhancive effects were established as the LD_{50} titer over this wide range of dosages for each agent and over a limited range as the LD_{100} titer.

Candida albicans. Fig. 4 illustrates the synergistic enhancive effect of cortisone and x-radiation upon infection of mice by a yeast,

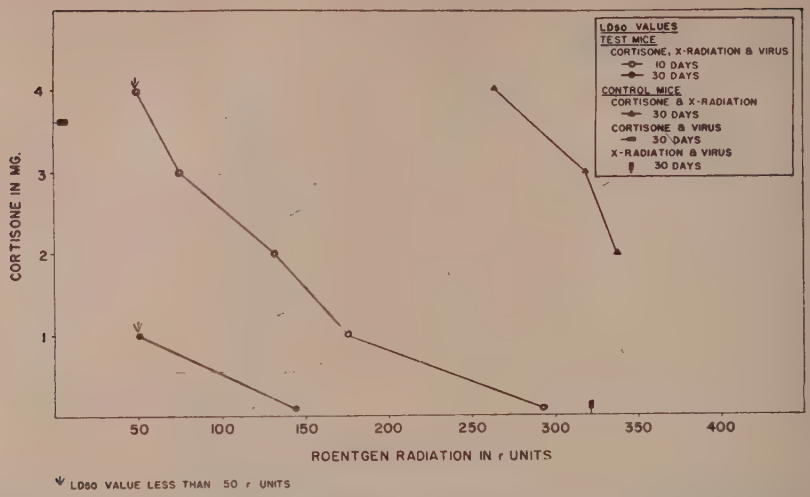

FIG. 3. The synergistic effect of cortisone and roentgen radiation on the pathogenicity of Coxsackie virus in resistant adult CFW mice as indicated by the LD_{50} value for a fixed dose of cortisone in combination with graded amounts of x-radiation.

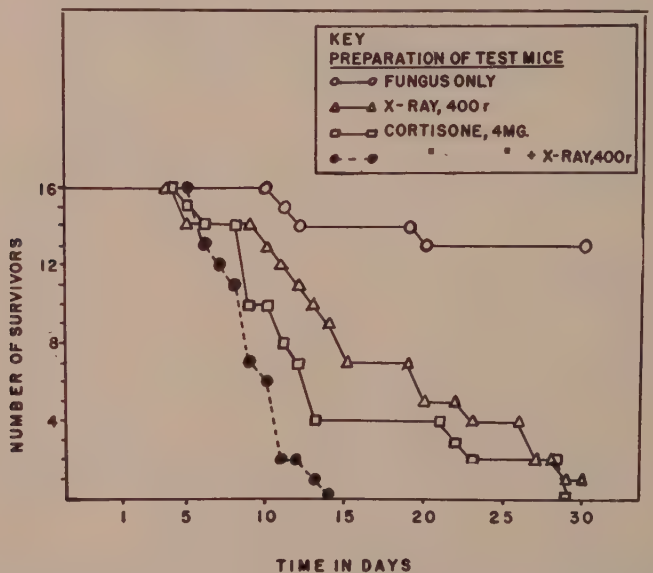

FIG. 4. The comparative effects of cortisone and x-radiation, singly and in combination, as agents for enhancement of the pathogenicity of *Candida albicans* for mice.

Candida albicans. It may be seen from Fig. 4 that a fulminant infection resulted in the mice which had been conditioned by cortisone, 4.0 mg, and x-radiation, 400 r. Twenty out of the 24 mice in the test group were dead within 6 days of infection and all 24 died within 9 days. In contrast to these findings, a) only 4 died of moniliasis of the 24 mice that received *Candida albicans* only; b) moderately effective enhancive effects were obtained from the use of x-radiation, 400 r, to result in death of 22 of 24 with an average

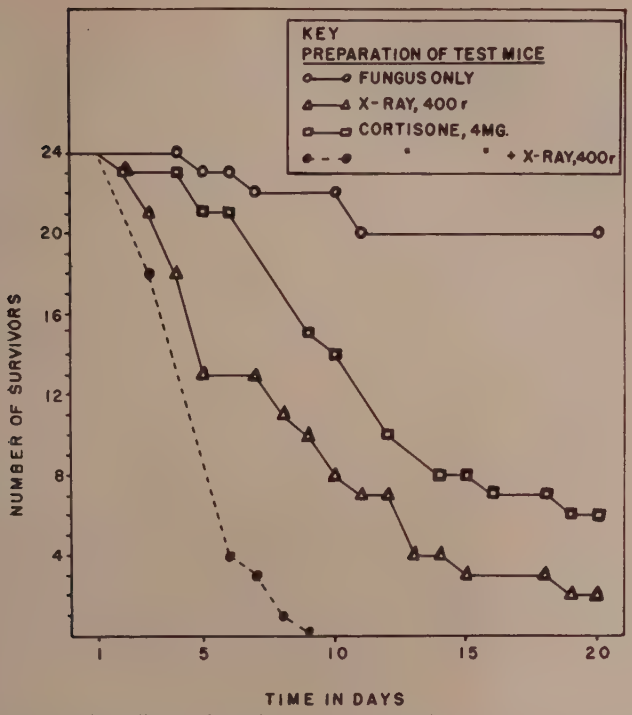

FIG. 5. The comparative effects of cortisone and x-radiation, singly and in combination, as agents for enhancement of the pathogenicity of *Blastomyces dermatitidis* for mice.

survival time to death of 7.5 days; and c) moderately effective enhancive effects resulted from cortisone, 4 mg, to kill 18 of 24 with a survival time to death of 11.7 days. Even though the lethality of the infection was markedly enhanced by the use of either cortisone or x-radiation, a prolongation in the time to death made either of these agents much less effective than the two in combination.

Blastomyces dermatitidis. Fig. 5 shows that the synergistic enhancive effect of the preparative treatment of mice by cortisone and x-radiation in combination upon infection by *Blastomyces dermatitidis* was essentially similar to that observed for *Candida albicans.* The treatment of mice by cortisone and x-radiation in combination before infection with *Blastomyces dermatitidis* resulted in death from blastomycosis of all 16 mice in the test group with an average survival time of 7.4 days. These findings are in contrast to the results that were obtained for the control group where death was limited to three of the 16 over a 30-day period. Finally, when preparative treatment was limited to either cortisone or x-radiation singly, an enhancive effect upon experimental blastomycosis similar to that observed for experimental moniliasis resulted in death of most of the recipients of the test inoculum over a period of 30 days, but the time to death was markedly prolonged.

Summary. A simple method is described for the production of rapidly progressive lethal infections. It consists of the administration of two readily available agents in combination, cortisone and x-radiation, to laboratory animals in preparation for test within the next 24 hours as recipients of a microorganism. Cortisone and x-radiation each over a wide dosage range act synergistically to potentiate the enhancive effect that may result from the employment of either agent singly. The result of this effect is a remarkable alteration

in the susceptibility of a test animal to infection by any of a variety of infectious agents, as evidenced by rapid weight loss, death and extensive histopathological lesions. The ready applicability of the method is illustrated in this paper by experiments that utilized four microbiological agents: poliomyelitis virus, a Coxsackie virus, *Candida albicans*, and *Blastomyces dermatitidis*. The experimental studies that employed these agents and the findings for other viruses, bacteria and fungi will be reported in detail elsewhere.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Cortisone-Induced Metastases of Adenocarcinoma in Mice.* (19544)

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In more than 200 routine transplantations of a mammary adenocarcinoma in C3H mice no metastases were found(1). Local growth of this transplantable tumor was inhibited by cortisone as was also adenocarcinoma in another strain(2). But a new and unexpected phenomenon not yet described, to our knowledge, has been found in our work with cortisone: inhibition of local tumoral growth by cortisone was accompanied by the appearance of multiple metastases. Our finding offers the opportunity to study the mechanism underlying production of metastases and the influence steroids may have on the spread of metastases in the body and on resistance against malignant growth. This is why a more detailed study with transplantable adenocarcinoma in C3H mice has been undertaken.

Material and methods. Male and female C3H mice about 2 months old, weighing 16 to 17 g in the first experiment, and 19 to 20 g in the other 2 were used. A 0.1 ml saline suspension of mammary adenocarcinoma K7‡ was injected into the left flank. Treatment

* Supported in part by a grant from the Rockefeller Foundation.

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‡ This adenocarcinoma appeared spontaneously in our C3H mice and was maintained, previous to the present work, by 51 transfers in 200 animals. For our technic see 1,3.

TABLE I. Weight of local growth and incidence of metastases in 20 C3H mice killed 24 to 41 days after inoculation of adenocarcinoma K7.

Group	Wt of local growth, avg and range (g)			Metastases incidence			
	24 days	37 days	41 days	24 days	37 days	41 days	Total
Not treated	5.6 (4.5-7.3)	7.9 (6.4-9.6)	15.7 (12.5-19)	0/3	0/3	0/2	0/8
Treated with cortisone	.6 (.5-.7)	.6 (.5-.6)	.7 (.7-.7)	0/4	0/6	2/2	2/12

TABLE II. Weight of Local Growth and Incidence of Metastases in 20 C3H Mice Which Died Spontaneously 36 to 76 Days after Inoculation.*

Group	Wt of local growth, avg and range (g)				Metastases incidence				
	36 days	43 days	68 days	76 days	36 days	43 days	68 days	76 days	Total
Not treated	6.6 (3.3-8.8)				0/7				0/7
Treated with cortisone	1 (.5-1.2)	.9 (.3-1.3)	1.2	1.8 (1.8-1.9)	2/4	4/6	1/1	1/2	8/13

* Two treated animals are still alive; not considered in Table II.

with cortisone began 8 to 9 days after inoculation when local growth of the tumor had reached about 7 mm in diameter; 0.5 mg of cortisone in 0.1 ml of saline were injected daily intramuscularly in the right thigh. In all experiments a group inoculated in the same way with the tumor but not injected with cortisone served as control. Some animals both of the experimental and control groups were killed at 24, 37, and 41 days after inoculation with the tumor suspension. The rest were left to survive until natural death. The animals were weighed every 3 days and growth of the tumors was recorded simultaneously. After death the weight of the tumor at the site of inoculation and the presence of metastases was registered. Tumors and metastases and organs (heart, liver, kidney, spleen, lymph nodes, adrenals, left thigh muscles) were fixed in 10% formalin and stained with hematoxylin and eosin.

Results. Increase of local tumoral growth was, in the treated animals, much less conspicuous than in the untreated ones; increase took place in treated animals only during the first days and remained practically stationary. At the end of the experiment (Tables I and II) the difference in weight of local growth between treated and untreated animals was remarkable. Average weight of the local growth in untreated animals was 5.6 to 15.7 g at 24 to 41 days after inoculation; in one animal growth reached at 41 days 19 g (Table I); average weight in treated animals was of 0.6 to 1.0 g, reaching 1.3 g in one animal (which died at 43 days), and 1.9 g in one animal (which died at 76 days; Table II).

A most conspicuous aspect in Tables I and II is the fact that there was not a single animal with metastases among the 15 untreated animals; on the contrary, there were 10 animals with metastases among the 25 animals treated with cortisone. This difference was not due to the longer survival of treated animals (Table II) as shown when considering only animals which were killed or died at 36 to 43 days (Tables I and II). There was not a single animal with metastases among the 12 untreated animals (36 to 41 d.); there were, however, 8 animals with metastases among 18 animals (36 to 43 d.) treated with cortisone. The metastases involved axillary, inguinal, mesenteric, and retroperitoneal nodes, the mediastinum, peritoneum, pleura, liver, spleen, kidney, lung, diaphragm, and the muscles of the thigh. Small nodules were present everywhere on the peritoneum except in the region contiguous to the local growth infiltrating the adjacent muscles of the abdominal wall.

Of considerable interest was the experiment

TABLE III. Weight of Local Growth and Incidence of Metastases in 18 C3H Mice Inoculated with a Suspension of Cortisone-Induced Metastases, or with a Suspension of Local Growth: Killed at 38 Days after Inoculation.*

Origin of suspension inoculated	Treatment	Wt of local growth, avg and range (g)	Metastases incidence
Metastases	0	9.4 (6.4-11.7)	0/6
"	Cortisone	.4 (.2-.6)	2/5
Local growth	0	9.1 (7.8-12.1)	0/4
" "	Cortisone	.6 (.2-1.5)	1/3

* Two animals died spontaneously at 36 and 38 days; not considered in Table III.

in which a suspension of cortisone-induced metastases was inoculated subcutaneously (Table III; axillary, inguinal and mesenteric lymphatic nodes were pooled when preparing the suspension). Local growth resulting from the inoculation of metastases was inhibited by cortisone as was local growth resulting from inoculation of the ordinary tumor. Local growth due to the inoculation of cortisone-induced metastases in a group of 6 animals was 6.4 to 11.7 g; when cortisone was given, the weight in a group of 5 animals was 0.2 to 0.6 g only (Table III). There were no metastases from local growth due to inoculation of cortisone-induced metastases; they, however, appeared when cortisone was given.

There were notable *histological* differences between treated and untreated tumors. Local growth in untreated animals consisted of polyhedric epithelial cells of great size; their nuclei were globular, rich in chromatin, with a great number of atypical forms and with mitoses; their cytoplasm was scarce and basophilic. The cells were gathered in irregular nodules, which were separated by a small amount of stroma. There were zones of central necrosis. Neighboring tissues were infiltrated by malignant cells. The tumor may be termed as encephaloid solid cancer, degree 4 of Broder's classification. In local growth of treated animals central necrosis was much more extensive. In the periphery there was a zone of variable thickness in which cells appeared dissociated and with clear necrobiosis such as degenerative changes of the nucleus, picnosis, caryorrhesis, and caryolysis. Numerous globular tumoral cells also were seen whose nuclear structure was apparently normal and whose cytoplasm was abundant and slightly eosinophilic; it was difficult to recognize them as epithelial due to the loss of normal relationship and to their histiocyte-like appearance; in the periphery these cells showed nuclear abnormalities. Mitoses appeared much less frequently than in tumors of untreated animals. The histological condition of the metastases was similar to that of local growth of the treated animals. Degenerative changes of liver and kidney were present in treated and untreated animals; a moderate degree of involution of the lymphoid-macrophage system was present in animals treated with cortisone.

In all groups but with one exception there was, at the end of the experiments, a decrease in *average body weight,* subtracting the weight of tumors. The decrease was 4.2 g in untreated animals, and 3.5 g in treated animals.

Discussion. It is clear that cortisone produced first a delay and later on an arrest of local growth. This effect was due either to a decrease of the mitotic activity, or to a change in the balance of the rate of production and destruction of cells. Both phenomena were evidently present since there was a decrease of mitotic activity and an increase of cellular necrosis. That cortisone induces metastases is, on the other hand, beyond any reasonable doubt. However, cortisone-induced metastases apparently suffer from cortisone the same cellular changes as local growth: both are histologically similar. Neoplastic active cells must be present in cortisone-induced metastases for, when inoculated subcutaneously, they behaved exactly the same as a suspension of a local growth of untreated animals.

Thus it becomes evident that cortisone has 2 different and antagonistic actions on resistance of C3H mice against transplanted adenocarcinoma K7: first, a protective antitumorigenic action through inhibition of local tu-

moral growth; and secondly, an action contrary to protection, through metastasizing or propagation of local growth in the body.

It is but logical to compare the described metastasizing action of cortisone to that of hyaluronidase which is known to favor propagation of infectious agents and tumoral cells (4,5); this action of the enzyme can be potentiated by estrogen, also a steroid hormone (6). There is the coincidence that both cortisone(7) and hyaluronidase(4,8) produce a decrease of mucopolysaccharides in connective tissues, but cortisone also acts on other constituents of the latter(7). The fundamental importance of this substance and of steroid hormones in general for the mesenchym is an established fact(9). All this would mean, though very hypothetically, that through the action of cortisone on connective tissue of the host, the nutrition of the tumor is impaired and at the same time the pathway is modified by which the malignant cells of the local growth can propagate in the body.

Modifications of the lymphatic system also may take part in antitumoral immunity(10).

Implication of the endocrine balance in tumorigenesis is beyond doubt(11). This does not mean that tumorigenic or antitumorigenic action of hormones on cells is always a direct one; however, the latter cannot be discarded. Indeed, a direct action of the steroid on the malignant cell was seemingly not sufficiently warranted by our work: cortisone-induced metastases when inoculated subcutaneously showed as to taking and tumoral development the same behavior as a suspension of local growth of untreated animals (Table III).

The real place of metastases in the complex of malignancy has been amply discussed in recent years(9). In the present paper metastases could be induced by a steroid which inhibits local growth from which these metastases start, and this finding gives new and striking evidence that the phenomenon of metastases is not simply one of the signs of malignancy as such, but a sign of a special phase of malignancy, possibly of malignancy in decay, as in our work, or of malignancy in an earlier phase of evolution, as in prostatic cancer and in goitrogen-induced thyroid tumors(12).

Summary. Local growth of transplantable adenocarcinoma K7 in C3H mice was arrested when cortisone was given. Metastases of transplanted adenocarcinoma were never observed in untreated animals. However, metastases appeared at about 5 to 6 weeks after transplantation in animals treated with cortisone. The longer the animals survived, the greater was the incidence of cortisone-induced metastases. The cortisone-induced metastases also were transplantable and produced local growth. But local growth due to transplantation of metastases did not produce metastases unless cortisone was given to the new host.

The authors acknowledge with pleasure the helpful criticism of Prof. Alexander Lipschutz during the preparation of this paper.

We are greatly indebted to Dr. Randolph T. Major of Merck and Co., N. Y. who provided the hormone ("cortone") used in these experiments.

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Received March 4, 1952. P.S.E.B.M., 1952, v80.

Neotetrazolium in Determination of Succinic Dehydrogenase Activity in the Ovary.* (19545)

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Tetrazolium salts as indicators of dehydrogenase activity in living cells are recent additions to the histochemical armamentarium(1). The compound used in the present study, pp' diphenylene bis 2-(3,5 diphenyl tetrazolium chloride) (neotetrazolium (NT)) is pale yellow in solution. It is converted to insoluble blue to black formazan at the site of active metabolic processes(2). The reduction of the tetrazolium salt by tissue slices appears to reflect the reductase or dehydrogenase activity of tissues and has been used to study such enzyme activity(3). If an excess of succinate is furnished as a substrate for the reaction, much of the formazan deposition is probably related to the presence of succinic dehydrogenase in the cells(4). Shelton and Schneider(5) found NT more useful than certain other tetrazolium compounds in localization of dehydrogenase activity. They felt that tetrazolium salts cannot be used for the demonstration of endogenous dehydrogenase activity in fresh tissue slices, but sites of succinic dehydrogenase activity can be localized in tissue sections. Using an homogenization method, Meyer and McShan(6) found a high level of succinic dehydrogenase in the corpora lutea of rat ovary during pregnancy. They felt that this high enzyme activity is correlated with function and growth of the corpus.

It seemed of interest to determine if formazan deposition takes place at sites of growth in the ovary. Blocks of ovaries of rabbits undergoing Friedman tests(7) were processed in neotetrazolium. It was felt that these tissues were associated with histologically demonstrable areas of rapid tissue proliferation. They should form a suitable basis for additional evaluation of the technic. In this study maximum formazan deposition corresponded well to sites of maximum growth.

Materials and methods. Nine virgin female rabbits were injected with urine from pregnant women and 9 with urine from nonpregnant women using a standard routine for the Friedman test(8), except that the second injection was given at a 24-hour interval. Forty-eight hours after the first injection fresh blocks from each ovary (about 3 mm in thickness) were separately placed in flasks containing 4.5 ml of 1% NT in 0.1 M phosphate in normal saline buffered to pH 7.4. To each flask, 0.5 ml of 0.3 M sodium succinate was added. Incubation with gentle agitation at 37°C for one hour followed. The blocks were then fixed in neutralized 10% formalin. Frozen sections (15 μ) from these blocks were mounted in gum arabic.

Results. At laparotomy, the ovaries of rabbits injected with urine from pregnant women had hemorrhagic follicles(7). The ovaries of rabbits injected with urine from nonpregnant women had cystic non-hemorrhagic follicles.

After incubation in NT all blocks acquired a deep purple color(2). In the sections, deposition of black or brown pigment was found. A light deposition was seen through the stroma in all ovaries. Heavily stained groups of interstitial cells were present in all ovaries. These were more concentrated and more prominent in Friedman negative ovaries (Fig. 1A). In all ovaries follicular cells around the ova had a strong staining reaction. The actual ova contained little or no pigment. The cystic follicles of the Friedman negative ovaries showed an intense deposition of fine pigment granules in the granulosa cells. The cell layers of the corpora hemorrhagica in the Friedman positive ovaries presented a much more prominent deposition of black pigment with larger granules than seen elsewhere (Fig. 1B).

* This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, P.H.S.

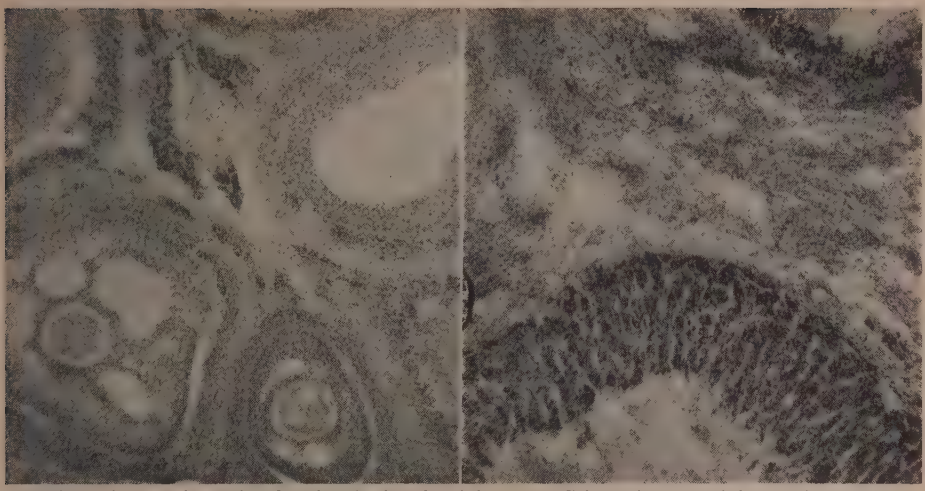

FIG. 1. Ovary of rabbit after incubation for 1 hr at 37°C in saline containing .9% neotetrazolium (NT) and .03 M sodium succinate. Reduced NT (formazan) deposited in cells. (A) Section from Friedman negative ovary with formazan granules outlining follicular cells, interstitial cells, and granulosa cells of cystic follicles (magnification 90 ×). (B) Section of Friedman positive ovary showing heavier deposition of formazan in wall of corpus hemorrhagicum (90 ×).

Occasionally cell layers adjacent to some of the largest hemorrhagic follicles had little or no pigment deposition. The larger cells of the corpora lutea contained finer and less prominent pigment deposition than seen in the corpora hemorrhagica.

Discussion. In general sites of formazan deposition correlated well with anticipated areas of tissue proliferation. Growth rate and metabolic rate are not parallel, but a rapidly proliferating tissue must be undergoing active metabolic processes. If NT is converted to formazan at the site of active metabolic processes(2), follicle cells of rabbit ovaries under the influence of follicle stimulating hormone in the urine of nonpregnant women should show moderate formazan deposition. In the present study this formazan deposition was found. Since the corpora hemorrhagica and corpora lutea of rabbit ovaries under the influence of urine from pregnant women become even larger, a much more active tissue proliferation was present. This was accompanied by the heavy deposition of larger pigment granules in the cell layers of the corpora hemorrhagica and corpora lutea. In this connection there is said to be an excellent correlation between the oxygen consumption of tissue as measured by the conventional Warburg technic and formazan deposition(1). The occasional failure of cell layers adjacent to some of the largest hemorrhages to show formazan deposition may result from: 1. Chemical interference by the blood in the hemorrhagic follicles. 2. Temporary diminution in dehydrogenase activity of adjacent tissue once hemorrhage has occurred.

It is interesting to consider the patterns of formazan deposition in relation to tissue growth in this series with analogous studies regarding human tumors(9) and growth of plant tissues(10).

Summary. Ovaries from rabbits injected with urine from pregnant and nonpregnant women were incubated in neotetrazolium with succinate. The granulosa cell layers of the cystic follicles in the ovaries of rabbits receiving urine from nonpregnant women showed moderate formazan deposition. The cell layers of the corpora hemorrhagica and corpora lutea in the ovaries of rabbits receiving urine from pregnant women showed much heavier deposition of larger formazan granules. In general, the pattern and degree of formazan deposition corresponded to areas of cellular proliferation.

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Received March 24, 1952. P.S.E.B.M., 1952, v80.

Absence of Eosinopenic Response to ACTH Administration by the Aerosol Route in Normal Subjects.* (19546)

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A number of investigators(1-5) have clearly demonstrated that the administration of ACTH in human subjects with unimpaired adrenal function produces a significant eosinopenia. This response has been employed as an index of adrenocortical function and in the assessment of the adrenocortical response to various forms of stress. In all instances, the hormone was administered either by the intramuscular or the intravenous route. The purpose of the present investigation was to study the effect of ACTH by the aerosol route upon the circulating eosinophils of normal individuals.

Plan of study. Six subjects (5 ♂ and one ♀), with ages ranging from 25 to 72, were selected. Two were free from any disease, one was affected with chronic bronchial asthma, but had been free from attacks for many months at the time of the experiment, and 3 had miscellaneous conditions (Table I). The entire group presented no signs of endocrine dysfunction, and all were permitted to carry on their usual routine during the period of investigation. An eosinophil count was taken on each of the subjects at 10 A.M., one hour after breakfast. This was considered the initial or control count. In order to check the accuracy of the procedure, several of the counts were repeated by another observer whose results agreed. The Henneman modification(6) of the Randolph method was employed in the following manner: 2 cc of venous blood were drawn and placed in an oxalate-containing tube. A white-cell pipette was then filled to the 1.0 mark with oxalated blood. Phloxine-propylene diluent was next drawn up to the 11 mark, and the pipette mechanically shaken for about 2 minutes. Both sides of 2 Max Levy double counting chambers were filled, and the eosinophils in 4 ruled areas (comprising 16 sq mm each) were counted after the expiration of one-half hour, to allow for settling. The total of the 4 areas was then multiplied by a factor, 0.78. Following the control count, 25 mg of ACTH dissolved in 1 cc of distilled water were injected intramuscularly and an eosinophil count was taken 4 hours later. After an elapse of several days to insure complete dissipation of the drug, control eosinophil counts were again taken as described above, and immediately afterwards, 25 mg of ACTH dissolved in 1 cc of distilled water were given to each subject by the aerosol route. In 3 instances a De Vilbis No. 40 hand nebulizer was used, and in the remaining 3, a Vaponefrin nebulizer, at-

* Acknowledgement is made to Armour Laboratories, Chicago, Ill., for supplying the ACTH.

TABLE I. Eosinophil Counts Before and 4 Hr After the Intramuscular Injection of 25 mg of ACTH (Aqueous).

Subject	Age	Diagnosis	Initial count	Final count	% change
P.	25	No disease	197	44	−73
S.	28	Asthma (free from symptoms)	248	70	−72
R.	32	Latent lues	125	25	−80
G.	46	No disease	184	42	−77
C.	69	Arteriosclerotic H.D.	150	71	−53
F.	72	" , gen.	225	81	−64

TABLE II. Eosinophil Counts on G. Before and 2, 3 and 4 Hr After Aerosol Administration of 25 mg of ACTH (in Propylene Glycol).

Time of count	Eosinophil count	% change
Before ACTH	180	—
2 hr after ACTH	200	+11
3 hr after ACTH	200	+11
4 hr after ACTH	220	+22

TABLE III. Eosinophil Counts Before and 4 Hr After Aerosol Administration of 25 mg of ACTH (Aqueous).

Subject	Initial count	Final count	% change
P.	175	181	+ 3
S.	225	215	— 4
R.	138	131	— 6
G.	156	206	+32
C.	144	194	+34
F.	169	169	0

tached by a rubber tubing to an oxygen tank which employed 5 to 6 liters of oxygen per minute. On one subject (G.) additional eosinophil counts were performed 2, 3, and 4 hours after the aerosol administration of 25 mg of ACTH dissolved in propylene glycol (Table II).

Results. The control or initial eosinophil counts were in conformity with what is to be expected in normal individuals. All subjects demonstrated a normal decrease (50% or over) in circulating eosinophils ranging from —53% to —80% following the intramuscular injection of ACTH (Table I). However, following the administration of the hormone dissolved either in aqueous solution or in propylene glycol by aerosol route, no significant drop in the circulating eosinophils was observed (Table III). A slight decrease of 4% occurred in S. and one of 6% in R., whereas all other subjects demonstrated variable increases. Such minimal alterations are frequently encountered in people irrespective of ACTH administration. A slight irritative cough was noted at the onset of the inhalation of the drug, and all subjects complained of a bitter taste. These symptoms were of short duration and in no way interfered with the completion of the test. The aqueous mixture was completely aerosolized in 5 minutes, whereas the procedure with ACTH dissolved in propylene glycol required 25 minutes.

Discussion. Hills, Forsham, and Finch(1) clearly demonstrated that a single intramuscular injection of 25 mg of ACTH causes a remarkably consistent eosinophil drop in normal subjects. They found that the decrease of the circulating eosinophils becomes apparent 2 hours after the injection of the drug, reaching its height at the end of 4 hours. They expressed this reduction as a percentage of the initial count and found it to be 50% or more in all instances of undisturbed adrenal cortical activity. Subsequently many other investigators confirmed this observation. Bonner and Homberger(7), employing the intravenous route of administration, obtained similar results with 5 mg of the hormone, and McIntosh and Holmes(8) with only 2.5 mg. The difference occurring with these 2 routes is explained by the fact that muscle tissue contains enzymes responsible for destroying the physiological activity of ACTH. The intramuscular route, therefore, does not appear to be the most efficient one for the administration of ACTH. The intravenous method, although requiring a much smaller dose, is cumbersome and is not entirely free from danger. An anaphylactic-like death has been reported from its use(7). The possibility of utilizing the aerosol route deserved investigation, therefore, since it would obviate these

difficulties, and, in addition, the hormone could be self-administered.

The administration of drugs by the aerosol route has long been an accepted mode of therapy. Many bronchodilators, antibiotics and the steroid hormone, cortisone, have been satisfactorily given by this method. The bronchopulmonary epithelium is highly vascular and offers an extensive area for absorption. When penicillin and other of the antibiotics are employed in this manner, blood levels comparable to those obtained when these agents are introduced either intramuscularly or intravenously are reached. There are some substances, however, which cannot penetrate this capillary barrier and are not absorbed by this means.

Rud(9), and Bonner and Homburger(7) demonstrated that simple fasting can produce a significant drop in the eosinophil levels in normal people. Hence, in order to overcome this physiological alteration, control counts were made shortly after breakfast.

The absence of adrenal insufficiency and the potency of the preparation used were indicated by previous response of the same subjects to intramuscular administration of the same lot of ACTH. Thus, it is apparent that ACTH, given by the aerosol route, is not absorbed.

Summary. Observations on 6 normal subjects showed that eosinopenia did not occur following aerosol administration of ACTH, indicating that ACTH is not absorbed from the bronchopulmonary tree.

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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Crystalline Material in Gastric Secretion. (19547)

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Following the report by Hollander(1) which described from light microscopy unidentified crystalline structures in dried smears of gastric juice, the attempt was made here to identify definitely the crystal molecule by means of electron and x-ray diffraction and to reproduce the dried, crystalline forms on electron microscope specimen screens. Human gastric secretion, obtained by Rehfuss tube from the stomachs of routine hospital patients was used in some experiments; specimens were also studied of mucus from Heidenhain pouch dogs obtained by topical stimulation with acetylcholine.*

Both the human and animal material were analyzed by electron and x-ray diffraction and found to be at least 90% NaCl. Table I shows the x-ray data. With the exception of a few low intensity lines the NaCl values were reproduced exactly by the mucus. The few extra lines were not identifiable. The only identified compound in both x-ray and electron diffraction was NaCl.

Small drops of the mucus were placed upon prepared electron microscope screens and dried slowly in air, and in the resultant electron micrographs all of the forms observed in the light microscope were discovered. Fig. 1, 2, 3, and 4 illustrate the appearances. Rela-

* Samples of dog secretion were obtained from Dr. Franklin Hollander, Mount Sinai Hospital, N. Y. City.

TABLE I. "d-values" and Intensities of Diffraction Patterns of Gastric Secretions.

Gastric secretion				NaCl reference	
X-ray		Electron		X-ray	
d	I	d	I	d	I
3.47 Å	.1				
3.27	.4	3.27 Å	.5	3.27 Å	.5
3.15	.7	3.15	.4	3.14	.4
2.82	1	2.82	1	2.82	1
2.22	.6	2.23	.3	2.21	.3
2	.9	2.01	.9	1.99	.9
1.82	.3	1.82	.9	1.81	.1
1.71	.2	1.72	.4	1.70	.4
1.63	.6	1.64	.6	1.63	.6
1.58	.2				
1.41	.5	1.41	.5	1.41	.5
				1.30	.2
		1.34	.7		
1.29	.2			1.28	.1
1.26	.6			1.26	.7
1.16	.6	1.15	.6	1.15	.6
1.11	.1				
1.09	.1			1.09	.3
1.05	.2				
.999	.5			.998	.5
.958	.1				
				.954	.2
.943	.5			.941	.5
.894	.5			.892	.5
.852	.4			.851	.5
.842	.1				
.817	.1			.815	.2
				.790	.2
.783	.2			.783	.4

tively large droplets produce the ferns and fronds of Fig. 1, 2, and 3. Small dilute droplets produced the single crystals of Fig. 4. Fig. 1 and 2 are reminiscent of parts of Fig. 4 and 5 in reference 1, and Fig. 3 is similar to parts of Fig. 2 in the same reference except for the larger magnification and superior resolving power of the electron microscope.

In Fig. 4 and others the single crystals appear to be cubic as would be expected of NaCl. Further evidence that the crystals are NaCl comes from electron bombardment experiments. When NaCl and some other crystals are bombarded by intense electron beams they evaporate in a characteristic manner, leaving a 3-dimensional residual envelope behind. Such envelopes of a large crystal and of some tiny crystals are seen in Fig. 4.

The evaporation of these crystals from gastric mucus progresses in a manner identical with that reported by Watson and Preuss(2) for NaCl. Fig. 5 shows before bombardment a particularly dense field in which no crystals are visible except in cracks and fissures in the deposited material (see arrows). Fig. 6 shows the same field after bombardment, and the crystals are now visible as residual envelopes. Even over the opaque part of the field the heat of the beam has driven off the crystal content and the locations of many large crystalline areas, previously invisible are demonstrated.

In addition to the crystalline component of the mucus there is also the non-crystalline, opaque substance which is nicely visible as background material in Fig. 7.

These observations authenticate the earlier beliefs of Henning and Norpoth(3) that the single crystals and fern-like aggregates were NaCl. The physical appearances which the crystalline material takes are not particularly significant and may be affected by such things as rate of drying, concentration of solution and presence of foreign organic matter: the chemical nature is not. The fern-like fronds are not specific for any particular crystalline substance but are often observed in biological specimens where foreign matter is present to affect the laying-down of the solid crystals. If the resolution and magnification are sufficiently increased, as in high resolution electron microscopy the ultimate single-crystal, cubic nature of such dried deposits of NaCl can always be demonstrated (Fig. 8). Certainly the effects cannot be attributed to stains used in the light microscope procedures because no stains are used in the electron microscopy although the same effects are produced.

The relatively high percentage of NaCl does not rule out the possibility of the presence of minute amounts of other crystalline material. The few additional x-ray lines demonstrated in the present work indicate that other crystals are indeed present. They might be gastric mucins. To identify them by x-ray diffraction would require concentration of the unknown with elimination of the NaCl.

Summary. Human and canine gastric secretion mounted upon electron microscope specimen screens is found to dry in fern-like patterns of tiny single crystals. These crystals evaporate under electron bombardment in a manner similar to NaCl. X-ray diffraction analysis of the dried secretions gives a strong

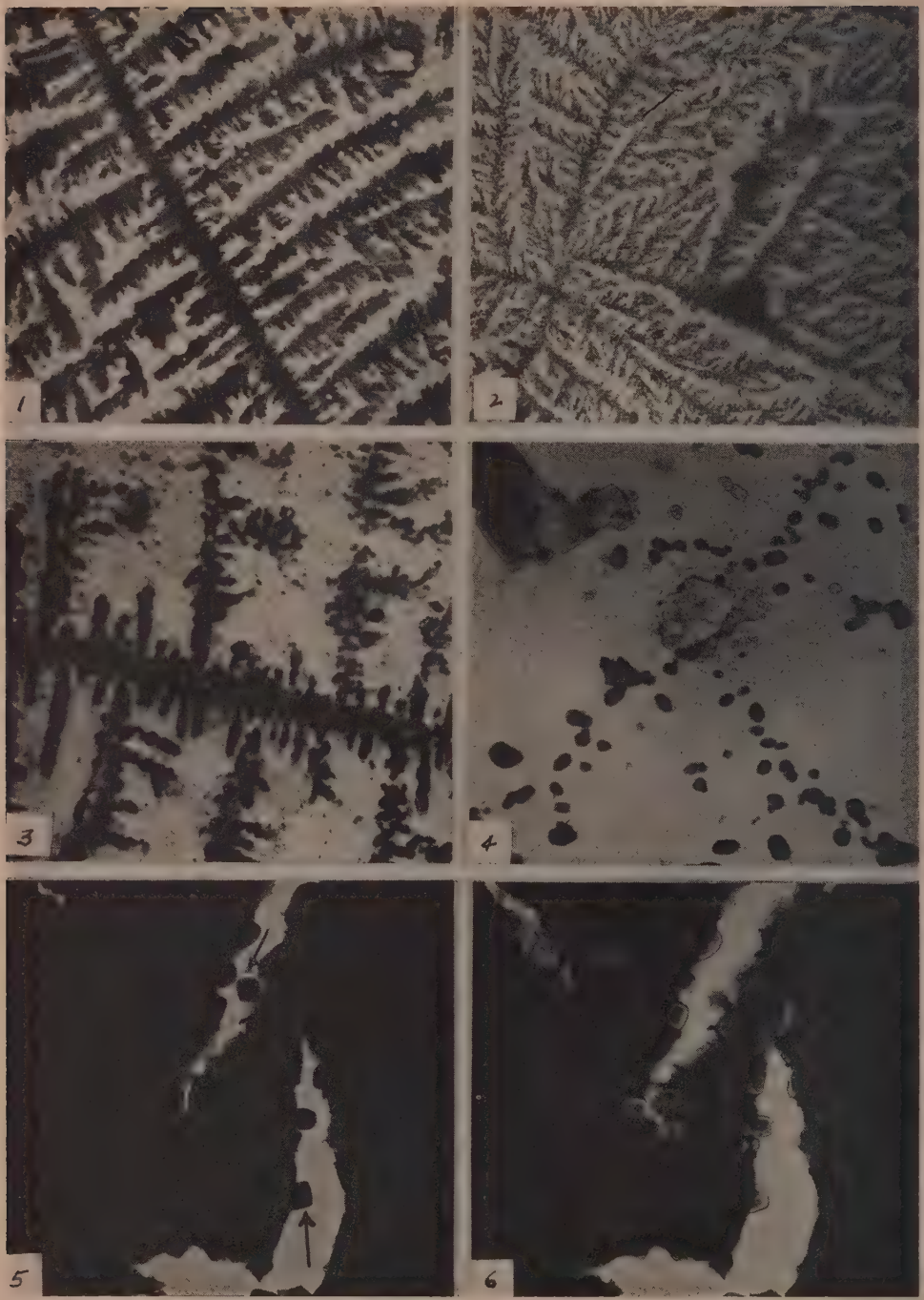

FIG. 1. Fern-like deposits of crystalline material in dried gastric secretions (human), ×7000.
FIG. 2. Same as Fig. 1, ×7000.

FIG. 3. " " " 2, ×5000.
FIG. 4. Single crystals in dried smears of gastric secretion (human), ×5000.
FIG. 5. A very thick deposit of dried gastric secretion (human) before electron bombardment, ×3000.
FIG. 6. The same field as Fig. 5 after electron bombardment, ×3000.

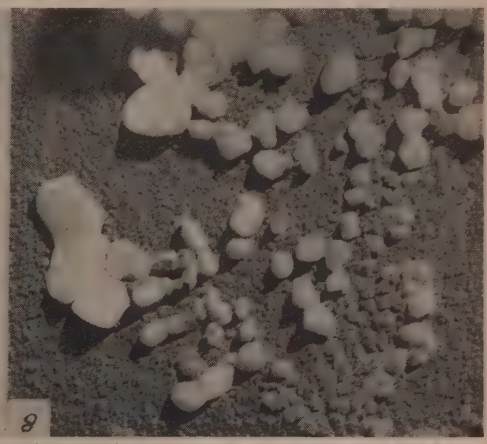

FIG. 7. A shadow cast preparation of gastric secretion (human) showing large NaCl crystals and amorphous background material, ×4200.
FIG. 8. A shadow cast preparation of NaCl itself. The field is part of a frond at high magnification to show the manner in which the fronds are formed of countless tiny crystals, ×25000.

NaCl identification with some weak unidentified lines also present.

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Received April 15, 1952. P.S.E.B.M., 1952, v80.

Diphenylacetyl-1,3-Indandione as a Potent Hypoprothrombinemic Agent. (19548)

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As a result of the therapeutic importance of anticoagulant drugs, the literature reveals a continuing search for compounds which will inhibit blood coagulation. A recent review by Seegers(1) includes a summary of the published information on prothrombinopenic substances, citing studies on indandione derivatives as hypoprothrombinemic drugs. A number of 1,3-indandiones, including two previously studied by Kabat, Stohlman and Smith (2), were prepared in these laboratories and submitted to us for evaluation as to their potential prothrombinopenic properties. Out of the group studied, 2-diphenylacetyl-1,3-indandione (U-1363; Dipaxin*) was a particularly potent hypoprothrombinemic substance.

The data establishing the above observation,

* Trademark, Upjohn Co.

and the results obtained upon further biologic evaluation of U-1363 as an oral anticoagulant, constitute the basis of this report.

Methods. Plasma prothrombin concentrations were estimated by a modified Quick(3) one-stage technic with Factor V (Ac-G) in the thromboplastic mixture. A standard thromboplastic mixture consisted of 12.5 ml of hog lung extract, 25 ml of hog serum (Ac-G) (4), 5 ml of 2.5% $CaCl_2$ solution, 0.5 ml of phenol, and distilled water q.s. to 100 ml. Aliquots were frozen and one was thawed daily during an investigation. Rabbits weighing approximately 3 kg were individually caged and fasted for 24 hours prior to the start of a prothrombinopenic study. From an ear vein was drawn 4 ml of blood into 0.4 ml of 1.34% sodium oxalate solution. The blood samples were centrifuged and the plasma assayed. Prothrombin times were determined on 100% plasma and after dilution with saline to 50%, 25%, and 12.5% plasma. Three determinations at each dilution were averaged. The values thus obtained for a given rabbit, before receiving any test compound, established the normal curve for that animal. Hence, each rabbit served as its own control. Subsequent prothrombin times were compared with the control values, and the existing prothrombin concentration calculated in terms of per cent of normal. Blood samples were obtained daily over the experimental period except in the chronic prothrombinopenic studies when determinations were made about every third day. Drug administrations were oral, either in capsules or by stomach tube. Food and water were consumed *ad lib.* after the initial bleeding. For the acute toxicity studies, U-1363 was administered by stomach tube in a 10% gum acacia suspension to rats and mice, and by capsule to rabbits. In the chronic toxicity evaluation, U-1363 was given by stomach tube daily for 14 days to rats and rabbits.

Results. Acute response. Table I presents the acute prothrombinopenic response of rabbits to single doses of 3,3′-methylenebis-4-hydroxycoumarin (Bishydroxycoumarin, U.S.P. XIV; Dicoumarol†), 3,3′-carboxymethylenebis-4-hydroxycoumarin ethyl ester (Ethyl biscoumacetate; Tromexan‡), and 17 analogues of indandione. Under the conditions of this test, U-1363 was the most potent. The data show that a pronounced and comparable prothrombin depletion resulted from 50 mg/kg of ethyl biscoumacetate, 10 mg/kg of bishydroxycoumarin, and only 0.05 mg/kg of U-1363. The entire acute response scatter curves for these 3 compounds at these dosages are presented in Fig. 1. With each of the drugs there was considerable individual variation in response. The average curves, however, demonstrate that after the single dose of ethyl biscoumacetate there was only a brief period of effectiveness before the prothrombin level returned to normal. In contrast, the small dose of U-1363 and relatively larger dose of bishydroxycoumarin resulted in

TABLE I. Evaluation of Compounds as Oral Hypoprothrombinemic Anticoagulants. Response to a single dose.

Compound (-1,3-indandione)	Dose, mg/kg	No. of rabbits	Lowest prothrombin values, (avg) % of normal
Ethyl biscoumacetate*	50	5	36
Bishydroxycoumarin*	10	10	38
2-diphenylacetyl-(U-1363)	.05	10	38
2-acetyl-	10	3	88
	50	2	60
2-phenylacetyl-	2	3	82
	50	2	50
2-triphenylacetyl-	5	3	86
2-pivalyl-	10	3	10
	5	3	18
	2	3	60
2-α,α-diphenylpropionyl-	50	2	50
2-α,β- "	50	2	50
2-β,β- "	50	2	45
2-isobutyryl-	50	3	40
2-isovaleryl-	50	3	40
2-α-phenyl-γ-methyl valeryl	50	2	50
2-benzoyl-	10	2	50
2-p-methoxybenzoyl-	10	3	100
	50	3	70
2-phenylazo-	10	3	88
2,2′-methylenebis-	10	3	90
2-(3′-keto-1′-indanylidene)-	10	2	100
	50	2	70

* Not indandiones.

† Trademark, Wisconsin Alumni Research Foundation.

‡ Trademark, Geigy Co.

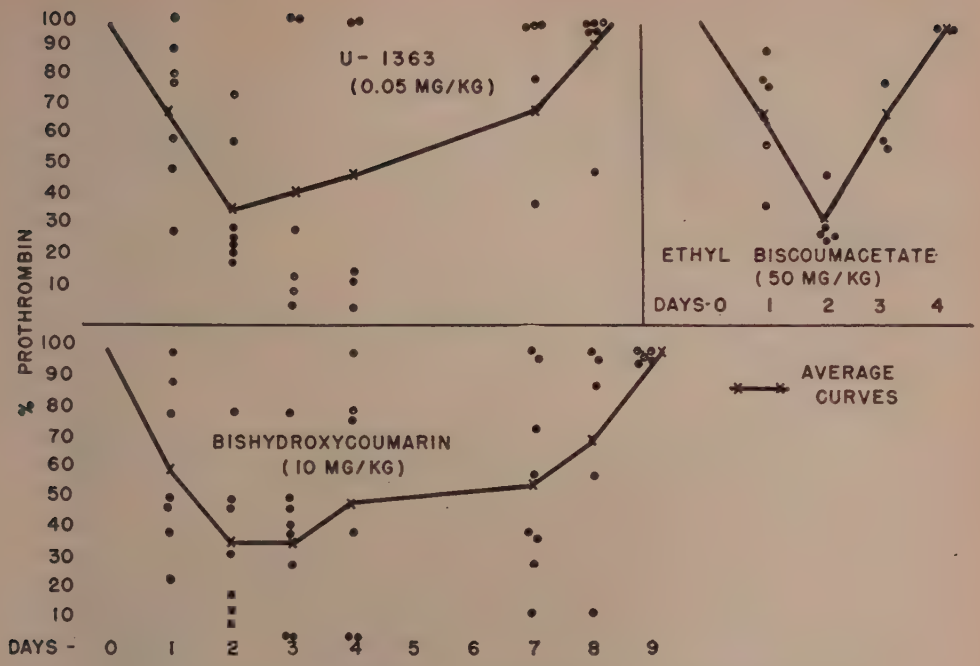

FIG. 1. Response of rabbits to a single oral dose of prothrombinopenic drugs.

more prolonged periods of prothrombin depletion.

Chronic response. Because U-1363 had demonstrated in single doses an acute hypoprothrombinemic potency, repeated doses were administered orally to 12 rabbits to determine the chronic regime which would lower the prothrombin level to around 30% of normal and maintain it at approximately that level for several weeks. Prothrombin determinations were made intermittently, usually every third day. Typical response curves are presented in Fig. 2 for four of the rabbits. Again, there was significant variation in the dose requirements of individual animals. Rabbits 397 and 394 maintained reasonably uniform low prothrombin concentrations on doses of 25 μg/kg administered, usually, every 1 or 2 days. Rabbit 390 was slightly more resistant, requiring several 50 μg/kg doses which were occasionally reduced to 25 μg/kg. Rabbit 389 responded to doses of 100 μg/kg, with a few days interspersed when only 50 μg/kg were needed.

Histopathologic observations. From this group of 12 rabbits, an animal was sacrificed at each of 4, 8, 10, 12, and 13 weeks for pathologic study. A gross examination was made, and sections of heart, kidney, liver, adrenal, lung, spleen, stomach and duodenum were taken for microscopic evidence of systemic toxicity. No such evidence was found, except for pulmonary petechia in moderate numbers in some of the rabbits, indicating a possible increase in capillary fragility. Hematologic studies revealed no effect on hemoglobin concentration or red and white cell count.

Acute and chronic toxicity. The acute oral LD_{50} for U-1363 was found to be 3 mg/kg for rats, 340 mg/kg for mice, and 35 mg/kg for rabbits. The number of animals used in these tests were 38, 75, and 21 respectively. The chronic oral LD_{50} for U-1363 was indicated as greater than 0.1 mg/kg/day with 12 rats. With 12 rabbits, a value of 0.25 mg/kg/day was obtained.

Antidote studies. U-1363 appeared to have potentialities as an oral hypoprothrombinemic anticoagulant. Experiments were therefore designed to ascertain if there might be an

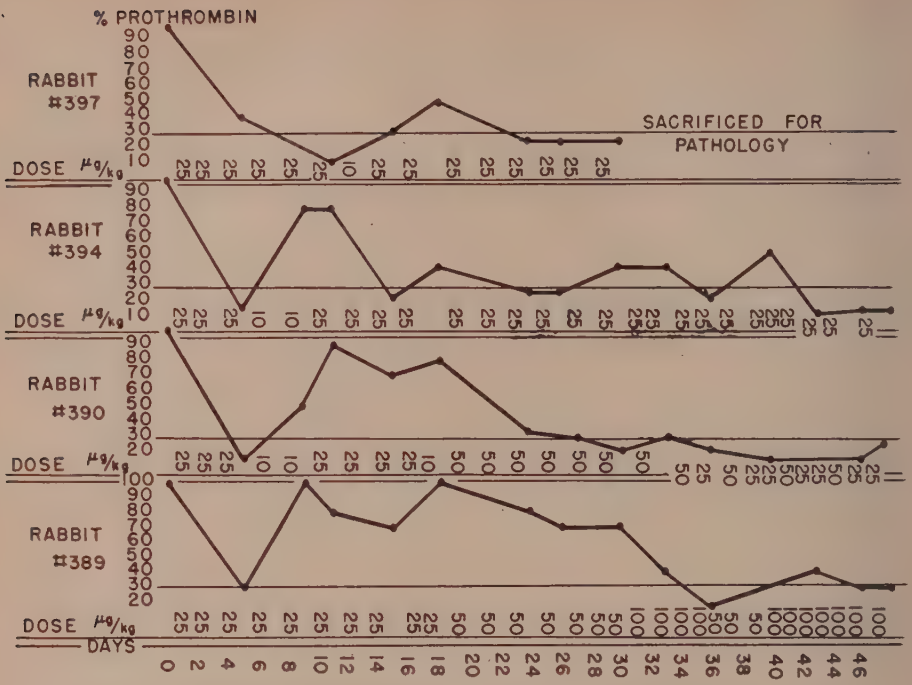

FIG. 2. Prothrombin response of rabbits to repeated oral administration of U-1363.

antidote for the hypoprothrombinemia produced by this drug. Thirty rabbits were given repeated doses of U-1363. After the last dose, prothrombin concentrations ranged from less than 10% (11 rabbits) to slightly above 30% (3 rabbits). Once hypoprothrombinemia was established, the administration of U-1363 was discontinued. Twelve of the animals, representing all degrees of depletion, served as controls receiving only food and water during the following 24 hours. At the end of this period, 6 controls showed either further prothrombin decreases or no improvement, 5 had slight to moderate increases. Only one of the group had returned to 100% of normal prothrombin concentration. The remaining 18 severely prothrombinopenic rabbits were administered amounts of vit. K_1 ranging from 20 to 400 mg per rabbit in 3 or 4 divided doses over the 24-hour period; 15 received the drug orally in capsules, 3 were injected intravenously with a fat emulsion of vit. K_1. At the end of the 24 hours, 13 had returned to 100% of normal prothrombin values, the remaining 5 had recovered to the relatively safe concentrations of 40 to 90% of normal. Thus vit. K_1, administered orally or intravenously in these doses, restored the prothrombin to normal within 24 hours after a U-1363 produced hypoprothrombinemia. In similar experiments, not reported in detail, it was found that a water-soluble vit. K analogue (tetrasodium 2-methyl-1, 4-naphtho-hydroquinone, diphosphate) had no influence on the hypoprothrombinemia produced in rabbits by U-1363.

Summary. Out of a group of compounds, including bishydroxycoumarin, ethyl biscoumacetate, and 17 analogues of indandione, tested in rabbits, 2-diphenylacetyl 1,3-indandione (U-1363) demonstrated the greatest hypoprothrombinemic potency. The prothrombinopenic response of rabbits to acute and chronic doses of U-1363 are reported. Histopathologic observations are reported for rabbits that were given U-1363 daily for several weeks. Acute and chronic toxicity data are reported for U-1363. Vit. K_1, but not a water-soluble vit. K analogue, is an

antidote to the hypoprothrombinemia produced by U-1363.

The authors wish to acknowledge the generous cooperation of the following associates from these laboratories: Drs. M. E. Speeter, D. G. Thomas and Mr. W. C. Anthony for preparing the indandione compounds; Dr. E. S. Feenstra for the histologic examinations; Mr. O. F. Swoap for the toxicity data; and Mrs. Russell Borst for technical assistance. The vit. K_1 emulsion was kindly furnished by Dr. F. J. Stare, Harvard University.

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Received April 16, 1952. P.S.E.B.M., 1952, v80.

Two Dimensional Paper Chromatography of Proteins. II. Application to Blood Plasma Fractions.* (19549)

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Franklin and Quastel(1) have reported that proteins may be separated by means of 2-dimensional filter paper chromatography; aqueous buffers and the ascent principle were used. These investigators converted the proteins into protein-hemin complexes prior to chromatography and identified the position of each protein-hemin complex by treating the paper with benzidine and hydrogen peroxide. All proteins, however, do not combine with hemin. The method is also limited by the intensely colored background which obscures the color given by the protein-hemin complex. In order to overcome this interference the patterns must be immediately photographed. While Hall and Wewalka(2) have criticized the method of Franklin and Quastel, the former authors concluded that proteins of dissimilar nature could be separated by chromatography. Feigl(3), as early as 1937, showed that dyes may be used for the detection of proteins on filter paper. Based on this principle, we have developed a convenient staining reagent containing the fluorescent dye, eosin, and methyl orange and presented other improvements concerning the 2-dimensional procedure(4).

*A preliminary report was presented before the American Society of Biological Chemists at the meeting of the Federation of Amer. Soc. for Exp. Biol., New York City, April 15, 1952.

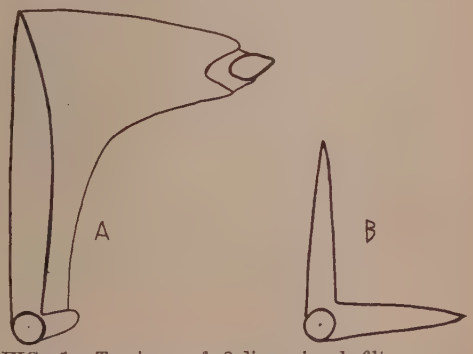

FIG. 1. Tracings of 2-dimensional filter paper chromatograms. A. = albumins, 120; B. = γ-globulins, 120. Quantities are in μg.

In the present paper we wish to describe the results obtained when our technic of 2-dimensional filter paper chromatography and staining was applied to human blood plasma albumins and γ-globulins fractions.

Experimental. The total protein applied in each experiment was 120 μg. The following solution of pH 6.0 was used as the developing agent in the first dimension: trisodium citrate (0.02 M), 9.25 ml (2 N) hydrochloric acid, and 50 g of sodium chloride per 5 l. The same buffer, after adjustment to pH 4.0, was used for dissolving the plasma proteins. Tartaric acid (2%) was employed in the second dimension.

In Fig. 1, "A" represents the tracing of the

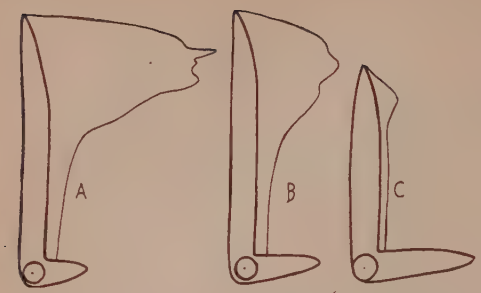

FIG. 2. Tracings of 2-dimensional filter paper chromatograms. A. = albumins, 100 + γ-globulins, 20; B. = albumins, 60 + γ-globulins, 60; C. = albumins, 20 + γ-globulins, 100. Quantities are in μg.

resulting pattern when 120 μg of Cohn's "albumins" of method 10(5), called "Fraction V," was applied to the paper. In "B" 120 μg of Cohn's "γ-globulins" of method 10(5), called Fraction II, was applied. In "A," the upper flag-like portion in the first dimension of the pattern is characteristic, under the conditions of the experiment, for the albumins fraction. In "B" the lower streak, in the second dimension, is typical for the γ-globulins fraction. In both instances there is movement in both dimensions.

These patterns are in harmony with the 3 patterns shown in Fig. 2. "A" in Fig. 2 represents a mixture of 100 μg of albumins and 20 μg of γ-globulins. "B" in Fig. 2 shows a mixture of 60 μg of albumins and 60 μg of γ-globulins. "C" represents 20 μg of albumins and 100 μg of γ-globulins. It may be seen that 20 μg quantities of either albumins or γ-globulins may readily be detected in the presence of 100 μg of the albumin fraction.

Summary. When albumins (Fraction V) were mixed with the γ-globulins (Fraction II) there occurred a typical separation of a portion of each plasma fraction. Our investigations indicate that 2-dimensional filter paper chromatography may have a definite value in protein chemistry, and in clinical research, even if complete separation of proteins is not possible at present.

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Received April 28, 1952. P.S.E.B.M., 1952, v80.

Gastric Ascorbic Acid in the Gastritic Rat.* (19550)

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Ascorbic acid, instrumental in the formation and maintenance of intercellular substance, may also play a role in gastrointestinal homeostasis. It has been demonstrated that ascorbic acid deficiency in guinea pigs results in a high percentage of gastric ulcers(1). Gastric cancer patients utilize more ascorbic acid than patients of other malignant or non-malignant diseases(2).

In the rat, in which abundant biosynthetic ascorbic acid is available, spontaneous and induced gastric disorder is rare(3). A eugenol-induced gastritis(4) is perhaps the only readily reproducible example of a gastric, glandular dyscrasia.

The purpose of the present investigation was to study the ascorbic acid in the tissue of the fore- and glandular stomachs of eugenol-treated, gastritic rats and comparable con-

* Aided by a grant from the Damon Runyon Memorial Fund.

† To be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Florida.

TABLE I. Effect of Eugenol Administration on Gastric and Adrenal Ascorbic Acid.

Group	No. of rats	Treatment	Chem. form.	Mean ascorbic acid — Stomach — Glandular mg %	Glandular μg	Forestomach mg %	Forestomach μg	Adrenals, mg %
I	10	Water st*	ox	2.2	24	3.3	11	15.8
			red	22.2	244	11.6	38	364.8
			tot	24.4§	268	14.9§	49	380.6§
II	7	Eugenol st	ox	2.9	32	2.7	9	16.6
			red	16.6	182	16.3	53	261.9
			tot	19.5	214	19	62	278.5
III	5	" di†	tot	24.8§	281	15.1§	50	266.6
IV	5	" gi‡	tot	19.4	213	18.8	62	290.4

* By stomach tube. † By duodenal inj. ‡ By gastric inj.
§ Comparison of corresponding totals from groups I and II and comparison of totals of groups III and IV are statistically significant. $P < .01$.

trols. The adrenals were also studied as a reference organ.

Methods. Male Sprague-Dawley-Holtzman rats, weighing 200-250 g received Purina Laboratory Chow and tap water *ad libitum*. Eugenol (4-allyl-2-methoxyphenol) as an aqueous 1.0% emulsion, or distilled water was administered daily in 3 ml doses for 7 days. The rats were divided into 4 groups according to the manner in which the eugenol emulsion or distilled water was introduced into the gastrointestinal tract. Group I: Distilled water was administered by stomach tube. Group II: Eugenol emulsion was administered by stomach tube. Group III: The rats were subjected to a) light ether anesthesia, b) a 2-3 cm abdominal incision, and c) injection of eugenol emulsion into the upper duodenal lumen. The musculature and skin were sutured with silk thread. This operative procedure was repeated daily for 7 days. Group IV: The rats were subjected to the same daily operative procedure as those of group III, but eugenol emulsion was injected into the gastric lumen. On the eighth day the animals were sacrificed by cervical dislocation. The forestomach, glandular stomach and adrenals were observed grossly, excised, weighed, and subjected to analysis for total and oxidized ascorbic acid(5). The value for reduced ascorbic acid was taken as the difference between the total and oxidized values.

Results and discussion. Weight loss was not observed in any animal. The results of the ascorbic acid determinations appear in Table I. The ascorbic acid in the stomach and adrenals was found predominantly in the reduced form. In the water-fed controls (I), the reduced ascorbic acid accounted for 91% of the total ascorbic acid found in the glandular stomach, 78% of that found in the forestomach, and 96% of that found in the adrenals. The concentration of total ascorbic acid was found to be significantly higher in the glandular stomach than in the forestomach. The glandular stomach of the rat is relatively less susceptible to spontaneous and induced dyscrasia than the forestomach(3). The possibility of a relationship between the pathological susceptibility of gastric tissue and the amount of reduced ascorbic acid present in the tissue must be considered.

Introduction of eugenol emulsion to the gastric lumen by stomach tube (II) resulted in a grossly recognizable gastritis which was distinctly absent in the water-fed controls. The total ascorbic acid concentration in the glandular stomach was significantly decreased in the gastritic rats (II), while in the forestomach the concentration was significantly increased. The total amount of ascorbic acid in the complete stomach of the gastritic group was 13% less than the total amount found in the water-fed controls. The percent of the total amount of ascorbic acid, found in the reduced form in the forestomach, glandular stomach, and adrenals of the gastritic group (II) was not significantly different from the percentage found in the water-fed controls.

Administration of eugenol emulsion by

stomach tube (II) resulted in a significant decrease in adrenal ascorbic acid. This reduction is suggestive of a stress phenomenon occurring after intestinal absorption. The possibility that the decrease of ascorbic acid in the stomach could also have been a systemic effect led us to place the eugenol directly in the duodenum. If the gastric effect observed in group II was systemic in nature, the ascorbic acid in the stomach should be decreased. Injection of eugenol emulsion into the duodenal lumen (III), did not evoke a grossly recognizable gastritis and the concentrations of ascorbic acid found in the fore- and glandular stomachs were almost identical to those of the water-fed controls. Injection of eugenol into the duodenum avoided direct gastric mucosal contact with the eugenol, but allowed intestinal absorption, as evidenced by the decrease in the adrenals. Since the adrenal decrease in ascorbic acid occurred in the absence of anesthesia and operative procedure, the reduction is probably related to the systemic presence of the eugenol.

The injection of eugenol emulsion into the gastric lumen (IV) resulted in a grossly recognizable gastritis and changes in gastric ascorbic acid concentration practically identical to those observed in the eugenol-fed, gastritic group, but significantly different from those in the duodenum-injected group. It may be concluded that the changes in the gastric ascorbic acid during a eugenol-induced gastritis, as well as the gastritis itself, are associated with the direct contact of the eugenol with the gastric mucosa, and not a systemic effect after gastrointestinal absorption.

Summary. Concentrations of reduced, oxidized, and total ascorbic acid in the forestomach, glandular stomach, and adrenals of the rat have been reported. Direct contact of a eugenol emulsion with the gastric mucosa resulted in a gastritis and a 13% decrease in the total gastric ascorbic acid. Systemic absorption of the eugenol, avoiding direct gastric contact, did not induce these changes. A decrease in adrenal ascorbic acid after eugenol administration suggests a systemic stress effect also associated with the presence of the eugenol, but distinct from the direct gastric effect.

The authors wish to express their sincere appreciation for helpful suggestions made by Dr. Michael Klein, and for the competent technical assistance of Lois C. Sumner and Dorothy E. Sawicki.

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Received April 29, 1952. P.S.E.B.M., 1952, v80.

The Action of Threonine in Inducing an Amino Acid Imbalance.* (19551)

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Of several amino acids reported to produce growth inhibition when fed in excess with a 9% casein ration, threonine appears to be one of the most effective in this respect(1). Since the addition of either niacin or tryptophan to this ration overcomes the inhibition produced by threonine, it appeared that the effect of the threonine might be due to an inhibition of the synthesis of tissue pyridine nucleotides from the already limiting amounts of tryptophan in the 9% casein ration. The

* Published with the approval of the Director of the Wisconsin Agric. Exp. Station. Supported in part by grants from the National Live Stock and Meat Board, Chicago, Ill., and the Nutrition Foundation, Inc., New York.

structural similarities between threonine and 3-hydroxyanthranilic acid could conceivably account for this inhibition in that threonine might act as an inhibitor of the enzyme system which converts 3-hydroxyanthranilic acid to the niacin structure.

Employing the technics worked out in this laboratory(2-7) for studying the relationship between dietary tryptophan and niacin and liver pyridine nucleotides (PN), the authors have investigated the effect of excess dietary threonine on these conversions. If threonine produces an imbalance by inhibiting the conversion of 3-hydroxyanthranilic acid to PN, the synthesis of PN from dietary tryptophan and 3-hydroxyanthranilic acid should be inhibited by threonine while that from niacin should not.

Experimental and results. In these studies 2 types of experiments were carried out. In one group of experiments, rats were first depleted of liver PN by feeding a non-protein ration. This ration was then supplemented with niacin, tryptophan, or 3-hydroxyanthranilic acid, with and without excess threonine in each case. After the period of supplementation liver PN concentrations were determined. In these experiments the rations were fed *ad libitum*. In the second group of experiments rats, depleted of liver PN, were fed a 9% casein ration with and without excess threonine to study the effects of threonine on liver PN concentrations using rations ordinarily employed in amino acid imbalance studies. In these experiments a force-feeding technic was employed to keep food consumption the same in all groups.

Ad libitum experiments. Male, weanling Sprague-Dawley rats were fed a non-protein ration for 14-21 days to deplete them of liver PN(3). The ration consisted of sucrose 87.5%, corn oil 5%, Salts IV(8) 4%, complete vitamin mix excluding niacin(9) 2%, and sulfasuxidine 1.5%. The sulfasuxidine was included to depress intestinal synthesis of niacin. After the depletion period, the rats were separated at random into 8 groups and fed the non-protein ration supplemented for the respective groups as follows: Group I, no supplement; Group II, 0.163% niacin; Group III, 0.270% DL-tryptophan; Group IV, 0.202% 3-hydroxyanthranilic acid; Group V, 1.76% DL-threonine; Group VI, 1.76% DL-threonine + 0.163% niacin; Group VII, 1.76% DL-threonine + 0.270% DL-tryptophan; and Group VIII, 1.76% DL-threonine + 0.202% 3-hydroxyanthranilic acid. The niacin, tryptophan, and 3-hydroxyanthranilic acid were fed at equimolar levels, and DL-threonine was included at 11 times the molar level of the other supplements to insure an excess of the inhibitor.

After the various groups were fed their respective supplemented rations for 4-7 days, the animals were sacrificed and liver PN determined by the method of Feigelson, Williams and Elvehjem(7). These experiments were repeated in their entirety. In the first series of experiments, in which a 21-day depletion period was employed, many of the animals died during the subsequent supplementation period. Therefore, in the second experiments, a 14-day depletion period was used in order to decrease the mortality during the supplementation period. The results of both studies were averaged together and are reported in Table I. The figures in the last column were calculated by subtracting the endogenous PN values, *i.e.*, the negative controls, from the total value of each of the other groups. Therefore, the actual PN contribution of each supplement in the presence and absence of threonine can be compared. From the results in the table it appears that in the absence of threonine liver PN is readily synthesized from niacin and tryptophan and to a smaller but significant extent from 3-hydroxyanthranilic acid. The poor contribution of the 3-hydroxyanthranilic acid may be due to its instability in the ration. When threonine was included with these supplements, liver PN was synthesized just as well from all the supplements as when threonine was omitted. Therefore, the hypothesis that threonine induces growth inhibition by interfering with the enzymatic synthesis of PN from tryptophan via 3-hydroxyanthranilic acid is not supported by these experiments.

Force-feeding experiments. In producing growth inhibition in typical imbalance experiments, excess threonine is usually fed with a 9% casein-sucrose ration + 0.2% cystine.

TABLE I. Effect of Threonine on Conversion of Niacin, Tryptophan, and 3-Hydroxyanthranilic Acid to Liver Pyridine Nucleotides (*Ad libitum* Experiments).

Group	No. of animals	Supplement, %	Pyridine nucleotides (γ/g liver)	Difference in supplemented groups and the negative control (γ/g liver)
I	17	0	625	
II	14	.163 niacin*	1165	540
III	14	.270 tryptophan*	960	335
IV	14	.202 3-hydroxyanthranilic acid*	725	100
V	17	1.76 DL-threonine	600	
VI	16	1.76 " + .163 niacin*	1250	650
VII	18	1.76 " + .270 DL-tryptophan*	920	320
VIII	14	1.76 " + .202 3-hydroxyanthranilic acid*	775	175

* These supplements were added at equimolar levels.

TABLE II. Effect of Threonine on Formation of Liver Pyridine Nucleotides from Tryptophan in a 9% Casein Ration (Force-Feeding Experiments).

Group	No. of rats	Ration	γ PN/g liver — Exp. 1	γ PN/g liver — Exp. 2	Avg of Exp. 1 and 2
I	16	9% casein basal	553	535	544 ± 23*
II	16	" " " + .180% DL-threonine	457	428	442 ± 25*

* Standard errors of the mean.

Therefore, it is possible that in the preceding experiments the levels of tryptophan and 3-hydroxyanthranilic acid were too high in comparison with the level of threonine and that the effects of the threonine were reversed by the supplements. In the following experiments a ration known to produce an amino acid imbalance with excess threonine was employed(1). In this ration the only source of tryptophan was that in the 9% casein, and no niacin was included. In order to overcome the effects of the low food intake on this ration when threonine is included, a force-feeding procedure was undertaken to insure comparable food intake in all groups.

Male, weanling Sprague-Dawley rats were depleted of liver PN by feeding a non-protein ration for 14 days. The animals were then divided into 2 groups and force-fed a 9% casein-sucrose ration + 0.2% cystine(1) with and without 0.180% DL-threonine, respectively, for 10 days. Since threonine is known to produce an imbalance when included in this ration, the effect of threonine on the production of liver PN from the tryptophan in the 9% casein ration could be studied. The rations were prepared as a slurry in water and force-fed by stomach tube in 3-4 portions daily. After the 10-day force-feeding period the rats were sacrificed and liver PN concentrations determined as before(8). The experiments were repeated in their entirety, and the results of both studies with the average of the two are reported in Table II. The low PN levels in the groups without threonine† indicate that the tryptophan in a 9% casein ration is extremely limiting for PN synthesis. However, the decrease observed when threonine was included in the ration indicates that this amino acid further limits the maintenance of liver PN. The hypothesis that threonine might induce the effects of an imbalance by inhibiting the conversion of tryptophan to PN is still possible though the authors feel that a better explanation is more probable in view of other effects observed in these experiments. In the course of the force-feeding experiments it was observed that gross utilization of the 9% casein ration was greatly inhibited by threonine.

† For normal weanling rats fed a good stock ration, liver PN is about 900-1000 γ/g liver.

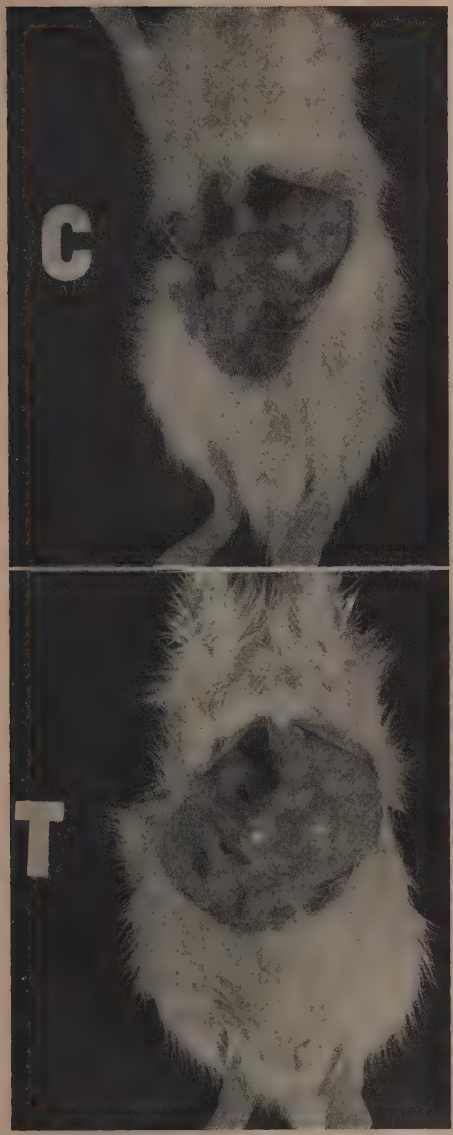

FIG. 1. Effect of excess dietary threonine on food retention in gastro-intestinal tract. Examples were chosen by different observers as the most typical of the two groups of rats. C = 9% casein control; T = 9% casein control + .180% DL-threonine.

This was demonstrated by the fact that the threonine-containing ration was only slowly digested and absorbed. When the animals were sacrificed for the PN determinations, very large amounts of the force-fed ration

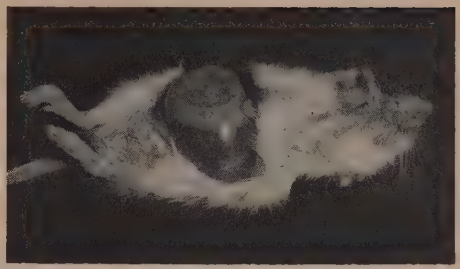

FIG. 2. A rat force-fed the 9% casein + .180% DL-threonine ration showing that the enlargement of the stomach is due mainly to unutilized food rather than to gas.

were found to be retained in the gastrointestinal tract, particularly in the stomach. This was true, however, only for the ration containing the excess threonine. Photographs of the most representative animal from each group, as chosen by a number of observers, are presented in Fig. 1. The marked retention of ration in the stomach of the threonine-fed animal can easily be observed. Another threonine-fed animal is shown in Fig. 2 in order to show that the enlargement is mainly due to food retention rather than to the presence of gas. From these results, therefore, it appears that threonine inhibits the digestive and absorptive processes when fed in excess in a 9% casein ration. The low liver PN values for this group might be explained by the decreased availability of the tryptophan in the ingested ration rather than by a threonine inhibition of the enzymatic synthesis of PN from tryptophan. This explanation appears more probable especially in view of the negative effect of threonine in the earlier experiments reported in this paper. In other results from this laboratory(10), it appears that if the protein is fed in the form of free amino acids rather than whole protein, threonine does not produce the typical amino acid imbalance syndrome in rats as measured by growth. Therefore, it appears from this evidence that excess dietary threonine induces the effects of the imbalance by interfering with the digestion and/or absorption of the intact dietary protein and thus limiting the availability of the tryptophan to the animal.

Summary. Experiments have been reported in which the function of threonine in produc-

ing an amino acid imbalance has been investigated. The hypothesis that threonine inhibits the conversion of tryptophan to tissue pyridine nucleotides via 3-hydroxyanthranilic acid is not strongly supported by our results. The results indicate, however, that excess dietary threonine markedly interferes with the digestion and absorption of the ration and that this is probably a better explanation for the action of excess threonine in producing growth inhibition in the rat.

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Received April 29, 1952. P.S.E.B.M., 1952, v80.

Gamma Globulin Passive Protection Tests in Mice Injected Intraperitoneally with MEF1 Poliomyelitis Virus.*† (19552)

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Human gamma globulin prepared from pooled adult plasma has been shown to possess substantial amounts of antibody against the Lansing strain (Type 2) of poliomyelitis virus (1). Similar observations have been made in connection with the other known types of poliomyelitis virus(2). Human gamma globulin when administered intraperitoneally to mice has been shown to afford some protection against the subsequent intracerebral inoculation of Lansing virus(3). More recently Bodian(4) demonstrated that in the case of monkeys 2.0 ml of gamma globulin per kg of body weight gave significant protection against the intramuscular inoculation of Lansing virus. The globulin and the virus were inoculated into the calf muscles of opposite legs. A protective effect could be demonstrated if the globulin was inoculated as much as 3 weeks before or 3 days after the injection of virus. Amounts of antibody sufficient to protect against the intramuscular injection of active virus did not interfere with the subsequent development of active immunity.

The recent demonstration that the suckling mouse-adapted MEF1 strain of poliomyelitis virus(5) can infect and cause the death of a significant proportion of mice aged 5-15 days when inoculated by the intraperitoneal route (6) has made available another laboratory animal which may be used for determining the efficacy of human gamma globulin in furnishing protection against the peripheral inoculation of a rodent-adapted strain of poliomyelitis virus. The present paper deals with the results of such an experiment.

Materials and methods. Two viruses belonging to the Lansing group (Type 2) were employed. The suckling mouse-adapted MEF1 strain (53rd passage material) was kindly furnished to us by Dr. Casals. The classical Lansing strain was obtained originally from Dr. Armstrong. Suckling mice of the CFW strain were used. The human gamma globulin was processed by E. R. Squibb (Lots No. 116-1 and No. 157-3) from pools of human plasma provided by the American National

* This paper presented in abridged form before the Society of American Bacteriologists, April 29, 1952. Abstract, *Bact. Proc.*, 1952, 103.

† Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Protective Effect of Gamma Globulin Against Intraperitoneal Inoculation of MEF1 Virus in 5-6-day-old Mice.

Group	Protective inoculation γ globulin	Mortality Exp. 1	Exp. 2	Exp. 3	Total	%	Diff./ S.E. diff.
I	0	18/35*	22/38	20/30	60/103	58.3	
II	.02 ml/lb	—	16/24	6/12	22/36	61.1	.3
III	.1	3/13	6/22	3/14	12/49	24.5	4.3
IV	.5	—	1/24	1/12	2/36	5.6	8.5
MEF1 inoculum:		Pool					
Passage No.		54, 55, 56	59	59			
Titer (i.c.)		$10^{-4.4}$	$10^{-3.7}$	$10^{-3.5}$			

* Numerator: No. of mice dying. Denominator: No. of mice inoculated.

TABLE II. Protective Effect of Gamma Globulin Against Intraperitoneal Inoculation of MEF1 Virus in 6-13-Day-Old Mice.

Group	Protective inoculation	Mortality Exp. 1	Exp. 2	Total	%	Diff./ S.E. diff.
I	0	45/57*	41/49	86/106	81.2	
II	Gelatin	26/32	—	26/32	81.2	.0
III	.02 ml/lb G.G.†	20/31	—	20/31	64.5	1.8
IV	.1 ml/lb G.G.†	7/29	10/35	17/64	26.6	8.1
V	.5 ml/lb G.G.†	9/35	—	9/35	25.7	6.7
MEF1 inoculum:						
Passage No.		61	61			
Titer		10^{-4}	$10^{-3.6}$			

* Numerator: No. of mice dying. Denominator: No. of mice inoculated.
† Gamma globulin.

Red Cross. In the first series of experiments litters of suckling mice 5-6 days old were pooled and redistributed at random after the average weight of the animals had been determined. Three groups of mice were injected intramuscularly with varying dilutions of gamma globulin so that the groups received 0.5 ml/lb, 0.1 ml/lb and 0.02 ml/lb of body weight, respectively. The dilutions were so adjusted that each mouse received the same volume (0.03 ml) of fluid. A fourth group received no injection and served as a control. Twenty-four hours later all mice were challenged by the intraperitoneal inoculation of 0.1 ml of a 10% suspension of MEF1 virus. Mice were inspected daily for 21 days for the presence of paralysis or death. In the second series of experiments 6-13-day-old suckling mice were used. Three groups received 0.5 ml, 0.1 ml, and 0.02 ml of gamma globulin per lb of body weight, respectively, while a fourth group served as uninoculated controls. An additional control group of mice inoculated with 0.03 ml of 1.5% gelatin solution was employed. Twenty-four hours later all the mice were challenged with MEF1 virus. Furthermore, the possible effects of the preliminary inoculation *per se* upon subsequent mortality was investigated by permitting corresponding groups of mice to go through the observation period unchallenged. In the third series of experiments 8-10-day-old mice were employed and the amounts of gamma globulin injected were 0.0125 ml/lb, 0.025 ml/lb, 0.05 ml/lb, and 0.1 ml/lb, respectively, for the 4 experimental groups. A fifth group received no protective inoculation. All mice in these 5 groups were challenged with MEF1 virus 24 hours later. A final group of mice received no preliminary inoculation and was not challenged with MEF1 virus.

Ten days after the close of each experiment of Groups I and II the surviving mice were challenged by the intracerebral inoculation of approximately 30 LD_{50} of Lansing virus in order to detect any evidence of the development of active immunity.

Results. Gamma globulin in concentrations

TABLE III. Effect of Injection of Gamma Globulin or Gelatin upon Subsequent Mortality Among 6-13-Day-Old Mice Not Injected with Virus.

Group	Protective inoculation	Mortality Exp. 1	Mortality Exp. 2	Mortality Total	Mortality %	Diff./S.E. diff.
I	0	13/51*	8/35	21/86	24.4	
II	Gelatin	11/31	—	11/31	35.5	1.1
III	.02 ml/lb G.G.†	7/32	—	7/32	21.9	.3
IV	.1 ml/lb G.G.†	5/31	1/29	6/60	10	2.4
V	.5 ml/lb G.G.†	7/30	—	7/30	23.3	.1

* Numerator: No. of mice dying. Denominator: No. of mice inoculated.
† Gamma globulin.

of 0.5 ml/lb and 0.1 ml/lb appeared to furnish significant protection in the 5-6-day-old mice employed in the first series of experiments (Table I). The respective mortalities for these groups was 5.6% and 24.5% as compared to 58.3% for the control group. No protection was observed in the group receiving 0.02 ml of gamma globulin per lb.

The second series of experiments employing mice 6-13 days of age gave similar results (Table II). Significant protection was conferred by gamma globulin injected in concentrations of 0.5 ml/lb and 0.1 ml/lb as compared to the controls. Mice receiving 0.02 ml of gamma globulin per lb showed a lower mortality rate as compared to control mice but the difference was not statistically significant. As was expected gelatin conferred no protection. No evidence was obtained that the protective inoculations alone increased the mortality in the injected mice as compared to the uninjected ones (Table III). In one instance (mice receiving 0.1 ml of gamma globulin per lb) the injected mice showed a significantly lower mortality rate as compared to the control mice. The explanation for this finding probably lies in non-specific factors affecting individual litters.

The third series of experiments employing mice 8-10 days old demonstrated again that 0.1 ml of gamma globulin per lb gave a high degree of protection. Half as much gamma globulin (0.05 ml/lb) conferred significant but less marked protection, while smaller amounts had no demonstrable protective effect.

In Tables V and VI are given the mortality rates of the various groups of survivors challenged by the intracerebral inoculation of Lansing virus one month after the first injection of virus, gamma globulin or gelatin. No protection was conferred by any of the injections of gamma globulin or gelatin alone. The mortality rate for each group was over 90%; the average for all mice was 94.1%. The control mice which had survived the injection of MEF1 virus although unprotected by gamma globulin showed a significantly reduced mortality (47.6%) as was expected. A significant reduction in mortality was observed also among the survivors in the group receiv-

TABLE IV. Protective Effect of Gamma Globulin Against Intraperitoneal Inoculation of MEF1 Virus in 8-10-Day-Old Mice.

Group	Protective inoculation	Virus	Mortality No.	Mortality %	Diff./S.E. diff.
I	0	+	44/47*	93.7	
II	.0125 ml/lb G.G.†	+	53/59	89.8	.74
III	.025 ml/lb G.G.†	+	52/58	89.7	.75
IV	.05 ml/lb G.G.†	+	39/55	70.9	3.2
V	.1 ml/lb G.G.†	+	21/54	38.9	7.3
VI	0 (controls)	0	15/47	31.9	8.1

MEF1 inoculum:
Passage No. 61
Titer (i.c.) $10^{-3.8}$

* Numerator: No. of mice dying.
Denominator: " " " inoculated.
† Gamma globulin.

TABLE V. Challenge of Control Mice with Lansing Virus.

Group	Protective inoculum	Mortality Ratio	Mortality %
I	0	62/65*	95.4
II	Gelatin	20/21	95.2
III	.02 ml/lb G.G.†	24/25	96
IV	.1 ml/lb G.G.†	47/52	90.4
V	.5 ml/lb G.G.†	22/23	95.7
Total		175/186	94.1

* Numerator: No. of mice dying.
Denominator: " " " inoculated.
† Gamma globulin.

TABLE VI. Challenge of Control Mice and MEF1 Inoculated Mice with Lansing Virus.

Group	Protective inoculation	Prev. MEF1 challenge	Mortality Ratio	Mortality %	Diff./ S.E. diff.
I	Controls	0	175/186*	94.1	
II	0	+	19/36	47.6	5.9
III	Gelatin	+	1/6		
IV	.02 ml/lb G.G.‡	+	6/10	60	—†
V	.1 ml/lb G.G.‡	+	25/47	53.2	5.5
VI	.5 ml/lb G.G.‡	+	21/26	80.8	1.7

* Numerator: No. of mice dying. Denominator: No. of mice inoculated.
† Numbers too small for comparison. ‡ Gamma globulin.

ing 0.1 ml/lb of gamma globulin prior to the intraperitoneal inoculation of MEF1 virus. The mortality rate among the survivors of the group receiving 0.5 ml of gamma globulin per lb was lower than that of the control group, but the difference was not statistically significant. The survivors among those receiving 0.02 ml of gamma globulin were too few to permit significant comparison.

Discussion. These results demonstrate that human gamma globulin administered to suckling mice in amounts as small as 0.05 ml/lb will confer significant protection against the intraperitoneal injection of the suckling mouse-adapted MEF1 virus 24 hours later. The duration of this passively conferred immunity has not been ascertained. In Bodian's observations in monkeys it was observed that an amount of gamma globulin adequate to protect against paralytic disease did not interfere with the development of active immunity. Our experiments confirm this when using 0.1 ml gamma globulin per lb, but although the number of mice involved was small the results suggest that 0.5 ml per lb may have prevented the development of an active immunity in some of the animals.

It will be noted that the mortality rate in unprotected mice challenged intraperitoneally with MEF1 virus varied from 58% to 93%. Conversely, a mortality rate of 24% to 32% was noted among the uninoculated control mice. These two extremes limit the present usefulness of this method as a means of testing the efficacy of biological or chemical substances in the prophylaxis of otherwise fatal infections with the MEF1 virus in suckling mice. Serial intraperitoneal passages of the agent in suckling mice, now in progress, may increase the virulence of the virus when inoculated by this route. The excessive mortality among uninoculated control mice can probably be reduced somewhat by more meticulous care of the individual litters. By such means there might be effected some reduction in the relatively large number of mice required in these experiments in order to obtain statistically significant results.

Summary. Suckling mice 5-13 days old were protected against the intraperitoneal inoculation of suckling mouse-adapted MEF1 virus by the intramuscular inoculation of human gamma globulin in amounts as small as 0.05 ml/lb 24 hours prior to the virus challenge. Human gamma globulin in an amount (0.1 ml/lb) adequate to protect against the intraperitoneal inoculation of the suckling mouse-adapted MEF1 virus did not prevent the development of active immunity against the subsequent intracerebral inoculation of Lansing virus.

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Received May 1, 1952. P.S.E.B.M., 1952, v80.

Effect of Amphenone "B"* on Adrenal and Thyroid Function of Adult Ovariectomized Rats.† (19553)

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Hertz, Allen, and Tullner(1,2) have shown that the administration of Amphenone "B" causes hypertrophy of the thyroid and adrenal glands of rats. This observation has been confirmed(3,4) but, thus far, the degree of function of these hypertrophied glands has been studied to only a limited extent(3,4). Hertz, Tullner, and Allen(2) have, however, expressed the suggestion that Amphenone "B" might be blocking the function of the adrenal gland much like thiouracil blocks the thyroid gland. With this in mind, experiments are here reported on the effect of the drug on thyroid function as measured by oxygen consumption, and on adrenal function as measured by electroshock seizure threshold (EST), thymus weight, and histological examination of the sectioned adrenal.

Methods and materials. Twenty-six adult rats ovariectomized 42 days prior to the time of drug administration, and 8 of which had been adrenalectomized 10 days previous to that time, were divided into 6 groups as shown in Table I. All rats received either tap water or 1% saline *ad libitum* in addition to pulverized stock diet (Purina Dog Chow) with or without the incorporation of Amphenone "B" (5) at a concentration of 0.6%. Inasmuch as saline is given advantageously to adrenalectomized rats, it was made available to 2 groups of animals (Table I) because of the possible decreased function of the adrenal(2). The Amphenone "B" as mixed in the food provided approximately 200 mg/kg/day to the average rat; the approximate average food consumption after the incorporation of the drug into the diet, was 33 g/kg body weight/day, as compared with a control intake of 50 g/kg body weight/day. For one week prior to the time of drug administration, control determinations of EST were made on all rats by the general technics previously reported by Woodbury, Sayers, Davenport, Goodman, and Cheng(6). The third control EST determination, made just before the first day of drug administration, was used as the control EST from which percentage changes were calculated for the following test period. During this period, the EST was determined 3 times per week. After 9 days of drug administration, the animals were fasted for 24 hours in preparation for oxygen consumption measurements. On the morning of these tests, 25 mg of Amphenone "B" in water solution (or a comparable volume of saline in the case of the controls) was injected subcutaneously into each test rat in lieu of the drug which the animal would normally have received in its food. The mean oxygen consumption of each rat was determined during 3 consecutive 15-minute periods(3). Immediately following the measurement of oxygen consumption, each rat was allowed stock diet (no drug) and water, or 1% saline, *ad libitum*, for a period of 30 minutes. Following this feeding period, the rats were put into individual metabolism cages, the post-prandial urine was collected for a 3-hour period, and a qualitative Fehling's test for urinary reducing substances was made thereon.‡

At the end of 17 days of drug administration, all rats were anesthetized with ether. After axillary blood had been collected from each for a blood glucose level determination (7), the rats were sacrificed and autopsied. The uteri as well as adrenal, thyroid, pituitary, and thymus glands were removed and weighed on a torsion balance. One adrenal gland from

*1,2-bis (p-Aminophenyl) - 2-methylpropanone-1 dihydrochloride.

† The authors wish to thank Dr. Roy Hertz and Dr. M. J. Allen, National Cancer Institute, Bethesda, Md., for valuable suggestions. Thanks, also, to Thomas L. Flanagan, Jr. of this laboratory, for the blood glucose determinations.

‡ Fehling's test using 12 drops of urine per 5 cc of total solution.

TABLE I. Effect of Amphenone "B" on Oxygen Consumption, Possible Glycosuria and Adrenal Sudanophilia of Ovariectomized Adult Rats.*

Group No.	No. of rats†	Description‡	Drinking fluid	Oxygen utilization§ (l/m²/hr)	"P"	Possible glycosuria‖	Histological adrenal sudanophilia
III	5	Ovarex control	Water	7 ± .6		1/4	1+
IV	5	" test	"	5.8 ± .6	.02	4/4	3+
VI	4	" control	Saline	6.5 ± .9		1/4	1+
V	4	" test	"	5.9 ± .7	.4	3/3	4+
II	4	Adrenex-ovarex control	"	5.8 ± .8		1/4	
I	4	" " test	"	5.3 ± .4	.3	2/2	

* All values expressed as mean ± S.D.

† During the experiment, 2 rats died in Group I, 1 in Group IV, and 1 was sacrificed in Group VI (Fig. 1-3).

‡ All test rats received Amphenone "B" concentration of .6% in pulverized stock diet for 17 days prior to autopsy.

§ Oxygen utilization of the rats was determined 10 days after onset of Amphenone "B" treatment. "P" was calculated by the "t" test(9).

‖ Qualitative post-prandial glycosuria expressed as No. of rats having detectable reducing substances in the urine (Fehling's test) over No. of rats tested.

each rat was placed in 10% neutral formalin. Following a >24-hour period of fixation, these adrenals were sectioned on a freezing microtome, and representative sections from each gland were stained en masse in a single staining rack. The stains employed were Sudan IV, Sudan IV counterstained with hematoxylin, and hematoxylin counterstained with phloxine(8).

Results. The results of the EST experiments indicate that the administration of Amphenone "B" caused an elevation of EST in adrenalectomized, ovariectomized rats (Fig. 1) and that the elevation was not as great in the Amphenone "B"-treated ovariectomized rats with adrenals (Fig. 2 and 3). In the rats that had adrenals, there was also a reversal of trend in EST concurrent with the 24-hour fast. This reversal in trend was continuous for the group receiving water (Fig. 2), but only temporary for the group receiving saline (Fig. 3).

The degree of trend reversal was in inverse proportion to the amount of sudanophilic ma-

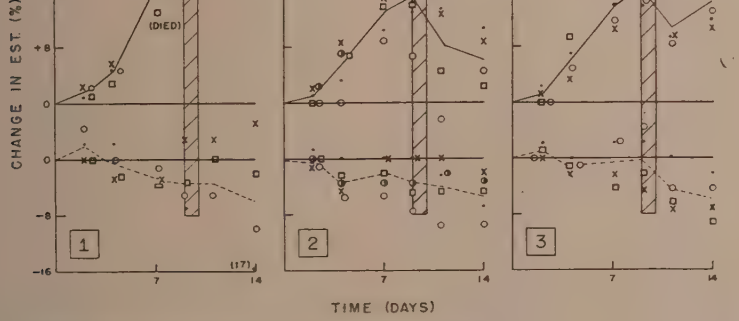

FIG. 1-3. Electroshock Seizure Threshold: Curves represent means; symbols represent EST of individual rats of each respective group; bar shows time of 24-hr fast. Upper curve, Amphenone "B"-treated with .6% in food. Lower curve, respective controls.

TABLE II. Effect of Amphenone "B" on Relative Weights of Endocrine Glands at Time of Autopsy of Ovariectomized Adult Rats.*

Group No.†	Final body wt (g)	Pituitary wt (mg/100 g)	Adrenal wt (mg/100 g)	Thymus wt (mg/100 g)	Thyroid wt (mg/100 g)	Uterine wt‡ (mg/100 g)
III	237 ± 24	4.4 ± 1	19 ± 7	223 ± 38	5.3 ± 1	11 ± 4
IV	185 ± 20	5.3 ± 1.4	33 ± 7	78 ± 19	14.2 ± 1.8	25 ± 6
VI	240 ± 13	5.3 ± .9	18 ± 1	209 ± 39	4.8 ± .7	9 ± 4
V	191 ± 9	5.3 ± .8	34 ± 9	128 ± 30	14.6 ± 2	19 ± 5
II	215 ± 39	4.5 ± .7	—	298 ± 25	8 ± 1	13 ± 2
I	190 ± 2.8	5.7 ± .3	—	176 ± 57	13.4 ± 3.5	23 ± 1

* All values expressed as mean ± S.D., with gland weights given as mg/100 g final body wt. See Table I for description of groups.

† Group numbers refer to same animal groups as those shown in Table I.

‡ Uterine portion weighed was only the body itself (often termed cervix) and not the cornua.

terial present in the adrenal (Table I), in that the group of rats having only a temporary reversal of the rising EST showed the greatest concentration of sudanophilic material.

As another measure of adrenal activity, it should be noted that the mean thymus weight (Table II) of the rats decreased according to the following order of animal groups: II, adrenalectomized, ovariectomized, control rats receiving saline; III, ovariectomized, control rats receiving water; VI, ovariectomized control rats receiving saline; I, adrenalectomized, ovariectomized test rats receiving saline; V, ovariectomized test rats receiving saline; and IV, ovariectomized test rats receiving water. The adrenal glands of all Amphenone "B"-treated rats were increased in weight (Table II).

The thyroid weight of all Amphenone "B"-treated rats was increased (Table II). However, only in the group of ovariectomized rats receiving water as the drinking fluid was there a statistically significant lowering of the oxygen consumption of the treated rats as compared with the control rats (Table I).

The uterine weight was increased consistently with Amphenone "B" treatment (Table II), whereas it is doubtful that the pituitary weight was affected.

Every animal receiving the drug showed a positive test for reducing substances in the urine, whereas only one-fourth of the control rats showed the same (Table I). Measurement of the blood glucose level showed that in no case was it above 150 mg% in either the control or Amphenone "B"-treated groups. It should be mentioned that Amphenone "B" failed to give a positive test when 10 mg were heated in 5 cc Fehling's solution; however, it is not known whether this positive reducing test is due to glucose, a metabolite of Amphenone "B", or some other undetermined substance.

By gross appearance, the adrenals of Amphenone "B"-treated rats were larger and more yellow in color than those of the normal animals. Histologically, there was a tremendous amount of sudanophilic material in all 3 layers of the adrenal cortex, but it was most evident in the *zona fasciculata*. In general, many of the cells of the cortex appeared to show a fatty metamorphosis-like change with small vacuoles observable in the cytoplasm but with little nuclear change. Adrenals from rats receiving Amphenone "B" with saline as the drinking fluid showed much greater changes of the type mentioned above than did those from rats receiving Amphenone "B" and water.

Discussion. The lower oxygen utilization of all Amphenone "B"-treated rats (statistically significant in one of 3 groups) would suggest a possible decrease in function of the thyroid gland. Related experiments have shown that Amphenone "B" is also a potent depressant of radioactive iodine uptake in the rat thyroid (4,10). On the other hand, the present evidence suggests an increase in function of the adrenal gland of Amphenone "B"-treated rats. Cortisone has been reported to depress the EST(6), and in this experiment, the presence of the adrenal in Amphenone "B"-treated rats

is associated with a smaller elevation of EST than that observed in Amphenone "B"-treated rats from which the adrenal had been removed (Fig. 1-3). This would suggest that increased endogenous cortisone production was counteracting the primary effect of the Amphenone "B". The super-imposed stress of the 24-hour fast may have caused an even greater activity of the adrenal as noted by reversal in trend of the rising EST of Amphenone "B"-treated rats (Fig. 1-3); this is a further suggestion of increased endogenous cortisone production.

If the degree of thymic involution is considered a measure of adrenal activity(11) then these data are in agreement with the EST data. For instance, of the Amphenone "B"-treated rats: the ovariectomized, adrenalectomized rats receiving saline showed the greatest elevation of EST as well as the greatest thymus weight; the ovariectomized rats receiving saline showed a lesser elevation of, and temporary reversal of EST, as well as lesser thymus weight; the ovariectomized rats receiving water showed the least elevation of, and continued reversal of EST, as well as smallest thymus weight.

The histological study of the adrenals showed the adrenals of Amphenone "B"-treated rats on saline to contain more sudanophilic material and to display a greater degree of fatty metamorphosis-like change than the glands of Amphenone "B"-treated rats receiving water. This suggests that either the adrenal desoxycorticosterone-like function may be implicated in the responses to Amphenone "B" administration, or that the increased sudanophilia is merely an indication of altered adrenal activity in response to the additional saline intake. Morphologically, it would appear as if the glands from the rats receiving water would be more functional than those of rats receiving saline and this is borne out by the EST test as well as the thymus weight measurements.

Glycosuria has been reported to follow the administration of cortisone to normal rats(12). Therefore, the possible glycosuria here observed might be the result of increased adrenal cortical activity which in turn would be in agreement with the EST and thymus weight studies. On the other hand, however, two adrenalectomized rats also showed an unexplained possible glycosuria.

The increase in uterine weight was similar to that previously reported by Hertz, Allen, and Tullner(1).

All data of this report point toward, or are consistent with, an increase in activity of the cortisone-like aspect of the adrenal in the Amphenone "B"-treated rats. However, it is unknown as to whether this is a direct action of the drug or whether it is mediated through the general alarm reaction.

Summary. When compared with appropriate controls, it appears as if rats treated with Amphenone "B" show a decrease in function of the hypertrophied thyroid gland as measured by oxygen consumption but a probable increase in cortisone-like function of the hypertrophied adrenal gland as measured by electroshock seizure threshold, thymus involution, reaction to 24-hour fast, and histological study of the adrenal.

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Received May 5, 1952. P.S.E.B.M., 1952, v80.

Effect of Hyaluronidase on Toxicity of Procaine and Tetracaine in Mice.* (19554)

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Hyaluronidase has been added to local anesthetic solutions used in regional anesthesia. This mucolytic enzyme will hydrolyze the hyaluronic acid barrier found in various tissues of the body and thereby promote diffusion of the injected fluid(1-7). Although anesthesia may be more complete under certain situations with the use of hyaluronidase some investigators have either observed or are of the opinion that there is an increased incidence of systemic toxic reactions from the local anesthetic when hyaluronidase is added (3,4,7). Therefore experiments were conducted to determine the effect of hyaluronidase on the systemic toxicity of procaine and tetracaine injected subcutaneously into mice.

Methods and materials. The LD_{50} of procaine and of tetracaine alone and in combination with hyaluronidase were determined by injecting the solutions subcutaneously into white mice of either sex and weighing 20 to 30 g. Fresh solutions of procaine hydrochloride (Merck) and tetracaine hydrochloride (Winthrop-Stearns, Inc.) were prepared in 0.9% sodium chloride solution. Fresh solutions of hyaluronidase† containing either 150 T.R.U. or viscosity units per ml were prepared in 0.9% sodium chloride solution. The hyaluronidase solution was mixed with the local anesthetic solution in the syringe immediately before injection. It was decided to keep the total volume of solution to be injected into each mouse constant irrespective of the weight of the mouse and the dose of the local anesthetic. Therefore each mouse received a total volume of 0.3 ml of solution of which 0.05 ml was either 0.9% sodium chloride solution or the hyaluronidase solution and the remaining 0.25 ml contained the required amount of local anesthetic agent. The final hyaluronidase concentration was 25 units per ml. Additional experiments were conducted to determine the influence of hyaluronidase on the blood level of procaine following its subcutaneous injection in mice. The mice were from the above mentioned animal stock. The preparation of the solutions and the method of injection were the same as that already mentioned. The amount of procaine in the blood was determined with the method of Brodie *et al.*(8) which is a modification of the procedure for determining sulfonamides. The mice were sacrificed by a blow on the head at various time intervals after the subcutaneous injection of 435 mg/kg of procaine hydrochloride alone or mixed with the hyaluronidase. Blood was removed by cardiac puncture and placed in a tube containing sodium oxalate. Within one minute, 0.2 ml of this blood was placed in a 50 ml glass-stoppered flask containing 0.5 ml of a 50% solution of sodium arsenite and 1.0 ml of distilled water. Three minutes later 1.0 ml of 0.8 M borate buffer (pH 9) and 20 ml of ethylene dichloride were added. The other modifications of Brodie *et al.* procedure were that 3.0 ml of 1 N HCl was used in place of 1.0 ml to extract the procaine from the ethylene dichloride and the volume of sodium nitrite and subsequent solutions was increased to 0.15 ml. The optical densities of the solutions were determined at 550 μ in a Model 6A Coleman Junior Spectrophotometer. Preliminary experiments demonstrated that the recovery of procaine when added to blood or plasma in amounts of 1.25 to 3.75 μg was $96 \pm 3\%$.

Results. Table I summarizes the results of the effect of hyaluronidase on the systemic toxicity of procaine injected subcutaneously in mice. The method of Litchfield and Wilcoxon(9) was used to calculate the LD_{50}. It was found that at the 19/20 confidence limits the systemic toxicity of procaine was

* This study was aided by a grant through Initiative 171, State of Washington.

† Wydase kindly supplied by Wyeth Inc., Philadelphia, Pa. and Alidase kindly supplied by G. D. Searle and Co., Chicago, Ill.

TABLE I. Summary of Effect of Hyaluronidase on Systemic Toxicity of Procaine Hydrochloride Injected Subcutaneously in Mice. Volume of solution injected .3 ml; hyaluronidase conc. .25 units/ml.

	Control					With hyaluronidase					
Dose, mg/kg	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	Probability of t value
370						10	0	10	7.1	± 3.3	
400	10	1	8	6.5	± 2.7	10	1	10	4.7	± 1.9	
435	15	0	7	5.2	± 2.4	15	7	13	4.8	± 9.2	
510	15	3	15	5.6	± 2.3	15	7	13	3.9	± 1.3	
550	10	4	9	5.6	± 3.1	10	5	10	4.6	± 2	
600	15	5	15	4.8	± 2.1	15	12	15	2.9	± 1	.2–.5
700	10	5	10	7	± 6.6	10	4	10	3.3	± 1.8	.1–.2
800	10	8	10	2.9	± 1.1	10	9	10	2.3	± .7	
900	10	9	10	.3	± 1.6	10	9	10	3	± 1.5	
LD_{50}	655 mg/kg					540 mg/kg					
5% limits	582-737 mg/kg					482-605 mg/kg					
	Potency ratio, 1.22 (significant)										

from 1.04 to 1.42 times greater when combined with hyaluronidase. However, when calculated at the 99/100 limits, the addition of hyaluronidase did not increase significantly the systemic toxicity of procaine. This LD_{50} for subcutaneously injected procaine is lower than that reported by Ting and Coon(10) but within the range reported by other investigators(11).

The time of onset of convulsions was recorded to the nearest one-fourth minute in an attempt to determine whether hyaluronidase increased the rate of absorption of the subcutaneously injected procaine. The appearance of convulsions was considered to be the most convenient sign of onset of systemic toxicity from procaine. Applying the "Student" t test at the 600 and 700 mg/kg dose levels it was observed that the addition of hyaluronidase did not change significantly the average time of onset of convulsions produced by the procaine.

One explanation whereby the combination of hyaluronidase with procaine did not increase its systemic toxicity could be due to a rapid rate of breakdown of procaine in the subcutaneous tissue. This problem was investigated by determining the procaine blood level of mice following the subcutaneous injection of procaine hydrochloride alone and in combination with hyaluronidase. The results are shown graphically in Fig. 1. This dose of procaine was selected with the opinion that the possible role of procaine destruction at the site of injection could be demonstrated more readily at a lower dose level. It was observed that procaine injected without hyaluronidase appeared in the blood of mice within one minute following the injection. The procaine blood level increased rapidly between the second and the eighth minute after the injection. Between the eighth and twentieth minutes, the level remained fairly constant except for the fall at

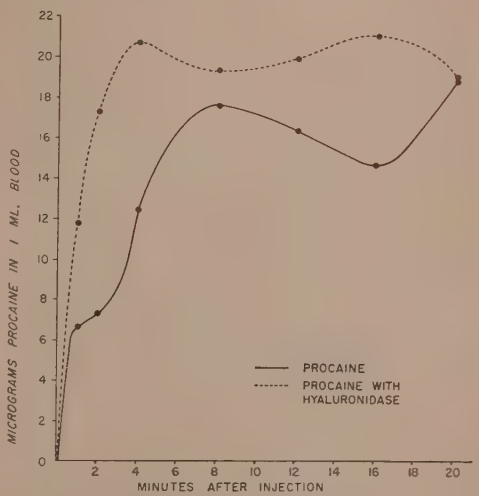

FIG. 1. Mean procaine blood level in mice following subcutaneous injection of 435 mg/kg of procaine hydrochloride alone and with hyaluronidase. Volume of sol. inj. .3 ml, hyaluronidase conc. 25 units/ml. Each point on curves are results from 3 to 5 mice. Probability of t value at: one min .1 to .2, two min .02 to .05, four min >.01, eight min .2 to .5, sixteen min .01 to .02.

TABLE II. Summary of Effect of Hyaluronidase on Systemic Toxicity of Tetracaine Hydrochloride Injected Subcutaneously in Mice. Volume of solution injected, .3 ml; hyaluronidase conc., 25 units/ml.

	Control					With hyaluronidase				
Dose, mg/kg	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.
20	10	0	7	4.6	± 1.4	10	1	9	3.5	± 1.2
30	10	1	10	3.3	± .9	10	4	10	3.3	± 2.5
40	10	5	10	3.4	± 1.5	10	6	10	2.5	± .6
50	10	6	10	3 *	± 1.1	10	7	10	2.1*	± .6
60	10	7	10	2.4	± .3	10	10	10	1.8	± .5
70	10	9	10	2.1	± .3					
LD_{50}	44 mg/kg					36 mg/kg				
5% limits	35-55 mg/kg					28-45 mg/kg				
	Potency ratio (not significant)									

* Probability of t value = >.5.

the 16-minute interval. Procaine injected with hyaluronidase resulted in a more rapid increase of the procaine blood levels. Using the "Student" t test, the procaine blood levels were significantly higher than the controls at the 2 and 4-minute periods. However, with the exception of the 16-minute period, there was no significant difference in the maximal blood procaine level attained in the 2 groups of animals.

It was noted during the experiments that there was no correlation between a given procaine blood level and the occurrence of convulsions.

After observing that the combination of hyaluronidase with procaine, increased significantly the systemic toxicity of procaine at the 5% probability, similar experiments were conducted to determine the effect of hyaluronidase on the systemic toxicity of tetracaine. This local anesthetic was selected because of its greater toxicity as compared to procaine. The statistical methods were the same as those used in the previous experiments. The results are summarized in Table II.

It was observed that the combination of hyaluronidase with tetracaine injected subcutaneously did not increase significantly the LD_{50} of tetracaine. Furthermore the addition of hyaluronidase did not significantly shorten the time for onset of convulsions.

The LD_{50} for tetracaine was the same as that reported by Fussganger *et al.*(12).

Summary. It was observed that only at the 19/20 probability limits did hyaluronidase increase the toxicity of procaine injected subcutaneously into mice. Hyaluronidase did not increase the toxicity of tetracaine within these same limits. From determinations of the procaine in blood of mice at various time intervals after its subcutaneous injection, it was observed that the procaine blood level increased rapidly beginning with the second minute and reached its peak at the eighth minute. The procaine blood level remained uniform between the eighth and twentieth minutes after injection. When hyaluronidase was mixed with the procaine injected subcutaneously there was a more rapid rate of increase of the procaine blood level than that of the control but the maximal procaine blood levels in the 2 groups were the same.

The authors wish to thank Dr. B. M. Bennett, Department of Public Health and Preventive Medicine, for aid in statistical analysis.

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Received May 6, 1952. P.S.E.B.M., 1952, v80.

Antifatty Liver Action of Papain and Ficin in Insulin-Treated, Depancreatized Dogs.* (19555)

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Previous work from this laboratory has shown that the fatty liver that develops in insulin-treated, depancreatized dogs can be prevented by the feeding of either methionine, choline, or hydrolysed casein(1,2). On the basis of these findings, it was proposed that the prevention of the fatty liver by raw pancreas, pancreatic juice and certain fractions derived from raw pancreas was the result of proteolytic enzymes furnished by the pancreatic substances(3). This view was later substantiated by the finding that crystalline trypsin, when added to the diet, also prevented the development of fatty livers(4).

The present report shows that prevention of fatty livers in depancreatized dogs can be achieved with plant preparations—papain and ficin—substances rich in proteolytic activity.

Experimental. The methods for assay of antifatty liver activity have been described elsewhere(5). Papain and ficin were fed twice daily to depancreatized dogs, for periods of 18-21 weeks, along with a diet consisting of 250 g of lean meat, 50 g of sucrose, 5 g of bone ash, and 1.5 g of salt mixture(4). Vitamin supplements(4) were fed once daily. Eight units of insulin were injected at each time of feeding. During the 3-4-week interval between pancreatectomy and the initiation of papain and ficin administration, the dogs were fed 25 g of raw pancreas with each meal in order to insure normal livers at the start of the assay period. At the end of the assay period, the dogs were anesthetized with nembutal. The entire liver was removed and thoroughly ground and mixed. Several samples were taken for the determination of total fatty acids(6).

Results. The fatty acid content of the livers of the control dogs fed no supplement ranged from 13.2 to 21.5% (Table I). The livers of the dogs fed papain were well within the normal range, *i.e.*, from 2.2 to 4.7%. Similar results were obtained with ficin-fed dogs; in these, the total fatty acid content of the liver varied from 1.6 to 2.5%. Throughout the course of the experiment the animals displayed a vigorous appetite. Ficin and papain did not prevent a loss in weight in these dogs.

Discussion. The capacity of papain to digest protein is well established(7), and its specificity, as determined on certain peptide linkages, is apparently similar to that of trypsin; indeed, papain is classified as a trypsinase (8). It is of interest to note that papain shows proteolytic activity over a wide range of temperature (10°-70°) and pH (3-12)(7). Although ficin has not been so extensively studied, it is nevertheless considered a papain-like enzyme(9).

Procedures for preventing the accumulation of fat in the liver of insulin-treated, depancreatized dogs can be classified into 1) those furnishing lipotropic substances in the free

* The work reported herein was supported by a contract from the Veterans Administration upon recommendation of the National Research Council's Committee on Veterans Medical Problems. We are indebted to Eli Lilly and Co. for the insulin used in this study.

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TABLE I. Effect of Papain and Ficin Feeding on Fatty Acid Content of Liver of 16 Insulin-Treated Depancreatized Dogs.

Pre-operative wt, kg	Final wt, kg	Supplement: Type	Supplement: Amt daily, g*	Supplement: Period fed, wk	Liver: Wt, g	Liver: Total fatty acids, % wet wt
9.5	7.2	None	—	20	670	21.5
6	5.5	"	—	24	580	13.3
7.9	6.5	"	—	19	310	37.3
9.2	8.2	"	—	12	720	17.2
13.1	9.2	Papain	.5	18	433	2.4
13.1	7.2	"	.5	18	443	4.5
12.8	9	"	1	21	433	3.1
12.8	11	"	1	21	382	2.5
10	9.4	"	1	21	432	3.1
9.7	6.3	"	1	21	356	3.5
13.8	10.7	"	4	21	630	4.7
6.4	6.6	"	4	20	463	2.2
14	8.7	"	4	20	495	2.8
8.3	6.6	Ficin	.5	21	400	1.6
9.1	7.8	"	.5	21	390	2.6
9.1	11.4	"	.5	18	420	2.5

* One-half of the amount recorded was fed with each meal.

form, and 2) those providing a means of liberating lipotropic substances from their bound form. There can be little doubt that the two plant enzymes, papain and ficin, act by replacing the loss of digestive capacity of the depancreatized dog. These results indicate that restoration of adequate digestive function, even with substances not specific to the animal organism, can prevent fat deposition in the liver.

Summary. It is shown that the development of fatty liver in insulin-maintained, depancreatized dogs can be prevented by the daily administration of the plant proteolytic enzymes, papain and ficin.

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Received May 6, 1952. P.S.E.B.M., 1952, v80.

Effect of β-propiolactone on Metabolism of *Pseudomonas aeruginosa* and Growth of Certain Fungi. (19556)

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β-propiolactone has been shown to be both bactericidal and virucidal and to have a relatively low toxicity(1-5). One of the organisms it is effective against, *Pseudomonas aeruginosa*, has been used in the following to study its possible mode of action. In aqueous solution the lactone hydrolyzes to β-hydroxpropionic acid, a fairly strong acid. This

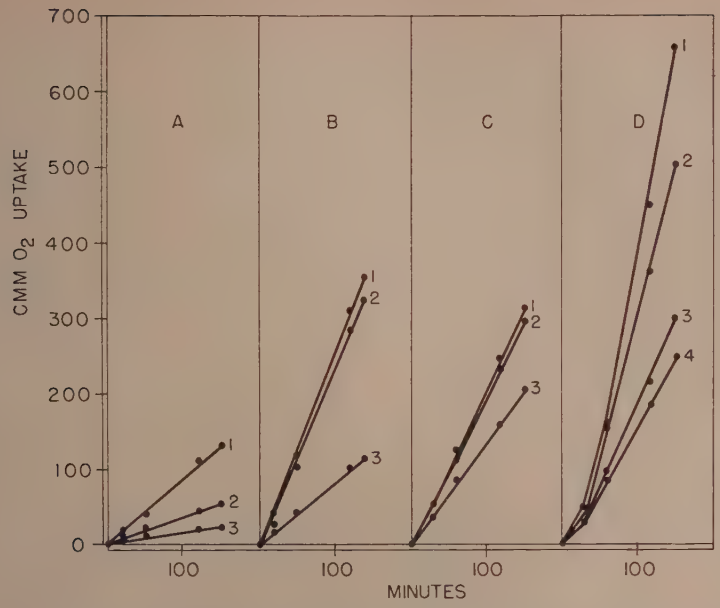

FIG. 1. The effect of β-propiolactone on oxygen uptake of *Pseudomonas aeruginosa* at pH 7.8 and 37°. Concentrations given are in mg/ml. Except in A the autorespiration has been subtracted. A1, .25 mg β-hydroxypropionic acid; A2, .25 mg lactone; A3, autorespiration. B1, 1 mg succinate; B2, + .25 mg β-hydroxypropionic acid; B3, + .25 mg lactone. C1, 1 mg succinate; C2, + .1 mg lactone; C3, + .15 mg lactone. D1, 1 mg succinate + .25 mg $(NH_4)_2SO_4$; D2, + .025 mg lactone; D3, + .1 mg lactone; D4, + .15 mg lactone.

property might make it a useful skin disinfectant, because the effect of the lactone would be augmented by the local increase in hydrogen on concentration. Accordingly some experiments on the effect of the lactone on the growth of certain fungi were undertaken.

Methods. To study the effect of the lactone on certain aspects of bacterial metabolism a strain of *Ps. aeruginosa* was used which was originally supplied by Dr. Koppers. This organism oxidizes acids of the Krebs' cycle with the concomitant assimilation of ammonia(6), it oxidizes amino acids(7), and forms adaptive enzymes for the oxidation of benzoic and p-hydroxybenzoic acids(8). It was grown as previously described(6), the cells washed by centrifugation, and placed in Warburg vessels with 0.05 M K-Na-phosphate buffer pH 7.8. The fluid volume was 2.0 ml. The lactone was obtained from the B. F. Goodrich Chemical Co.

Results. It was first necessary to determine whether the lactone or the product of its hydrolysis was the effective agent. Accordingly, the lactone in a concentration of 1.0 mg/ml in buffer was allowed to stand at room temperature for 24 hours before it was added to the bacterial suspension and its effect compared with a freshly prepared solution. The results are shown in Fig. 1, A & B. The lactone in a concentration of 0.25 mg/ml almost completely inhibited the oxidation of succinate whereas the hydrolyzed product was without effect. Moreover, the latter was oxidized slowly by the bacteria. The lactone increased the oxygen uptake of the bacteria to a very small extent. This indicates that it was fixed by the cells and thus not available for hydrolysis since the free lactone is rapidly hydrolyzed in alkaline solutions. The buffer prevented any pH change resulting from the hydrolysis of the small amounts of lactone used.

When succinate is oxidized in the presence of added ammonia the rate but not the amount of oxygen uptake is increased(6). Certain

antibiotics in low concentrations have no effect on the oxidation of succinate alone but inhibit the increased rate in the presence of added ammonia thereby inhibiting its assimilation(6,7). Fig. 1, C & D, shows that the lactone had a similar effect. As little as 0.025 mg/ml inhibited the increased oxidation rate in the presence of ammonia but had no effect on the rate of oxidation of succinate alone. As the concentration of lactone was increased, inhibition of the succinate oxidation occurred.

The previous addition of succinate protected the enzymes against the lactone. In this experiment, with 0.2 mg of lactone/ml at the end of 2 hours the succinate oxidation was inhibited 49%, and in the presence of ammonia 61%. The lactone was added 10 minutes before the succinate. When the succinate was added 10 minutes before the lactone there was no inhibition of its oxidation, and only a 29% inhibition in the presence of added ammonia. This effect occurred with other substrates and is thus non-specific. The lactone has little effect on the succinoxidase of animal origin.

Of all the amino acids tried, the oxidation of phenylalanine is most sensitive to the action of several antibiotics(7). The possible reason for this is that it utilizes high energy compounds during one stage of its oxidation. The lactone showed a similar selectivity. 0.05 mg/ml inhibited the oxidation of phenylalanine 50%, tyrosine 23%, and had no effect on the oxidation of alanine and serine. It did, however, inhibit the oxidation of propionic acid 52%. Finally, the oxidation of p-hydroxybenzoic acid which requires the formation of an adaptive enzyme and is inhibited by a number of antibiotics(8) was inhibited 36% by 0.05 mg/ml of the lactone. In this organism, therefore, it appears that the lactone has an action similar to that of streptomycin, aureomycin, and certain other antibiotics.

Trichophyton mentagrophytes and *Epidermophyton floccosum* obtained from the Department of Bacteriology, Duke Hospital, were grown on Sabouraud's medium at pH 5.6 and the plates were incubated at room temperature for 10 days. At this pH the hydrolysis of the lactone is delayed and the acidity of the medium remained constant. 1:1000 concentration inhibited growth of these 2 organisms completely as well as that of a 7-day-old yeast culture of *B. dermatitidis* incubated at 37°C. Furthermore, when scrapings were made from these plates at the end of 4 days and streaked on fresh plates without the drug, no growth occurred. A 1:5000 concentration inhibited growth approximately 50%.

Discussion. Because β-propiolactone is fungistatic as well as bacteriostatic it may be useful as a surface disinfectant. It would be necessary to store and apply it in a non-aqueous medium. Once in contact with the skin some of the lactone would be taken up by the organisms and the remainder would hydrolyze slowly, thereby keeping the area acid. The combined effect of the lactone and acidity should inhibit growth. Because of its low toxicity, it could be applied frequently without danger of systemic effects from absorption through the skin.

Summary. 1. β-propiolactone inhibits the oxidative metabolism of a strain of *Pseudomonas aeruginosa*. The mechanism of the inhibition appears similar to that of streptomycin, aureomycin, and certain other antibiotics. 2. Its hydrolytic product, β-hydroxypropionic acid, is without effect. 3. In low concentrations it inhibits the growth of *Trichophyton mentagrophytes, Epidermophyton floccosum,* and *Blastomyces dermatitidis*. 4. Its possible use as a surface disinfectant is discussed.

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Received April 25, 1952. P.S.E.B.M., 1952, v80.

Pancreatic Fibrosis in Ducks on a Nutritional Basis.* (19557)

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Pathological changes occurring in the acinar tissue of the pancreas have been discussed recently by Wallace *et al.*(1), Baggenstoss (2), Davies(3), and Véghelyi *et al.*(4). In a series of 200 autopsies(1) on cases excluding diabetes mellitus, acute pancreatic necrosis and carcinoma of the pancreas, fibrosis of the pancreas was present in 42%, being more predominant in older age groups. Baggenstoss (2) reviewed 270 cases of uremia and frequently observed a "remarkable degree of dilation of the acini, flattening of the lining epithelial cells, and inspissation of secretion . . . neither age, sex nor degree or duration of azotemia appeared to play a significant role in the production of the lesion. In the control series of 200 cases in which uremia did not occur, the lesion was present in mild or moderate degree in 40 cases (20%)." In some cases (rarely) an entire acinus was destroyed and replaced by fibroblasts and reticulum fibers. Davies(3) reported the essential pathology of the nutritional disorder, Kwashiorkor, which is widespread in Africa. In the pancreas, "The cells undergo hyaline changes, the tubules are dilated; and a periacinar, intratubular, and perilobular fibrosis begins . . . The effect is that the acinar tissue is broken up by the fibrosis and lobular outlines are shown up by dense bands of fibrous tissue." Véghelyi(4) has described a progressive decrease and apparent cessation of the excretory function of the pancreas in infants nourished by a diet lacking in animal protein. Histologic observations on the pancreas of some of these infants revealed "that the acinar cells became small, atrophic and angular and separated from each other. A considerable part of the cytoplasm disappeared and only few, if any, secretory granules were visible. . . . The advanced stage of the pancreatic disorder observed in several infants who died after 8 to 12 weeks was distinct fibrosis around the lobules and invasion and intersection of the parenchyma by fine bands of connective tissue. In no instance were the islets seen to be affected." These authors(4) were able to produce similar pancreatic lesions in rats fed diets containing low quality protein. Other reports (5,6) describe pancreatic fibrosis in experimental animals similar to that observed in man.

In recent nutritional studies(7) fibrosis of the pancreas was observed in ducks fed a purified diet in which the corn starch was replaced by sucrose. It has been suggested that this lesion is the result of a nutritional deficiency(8).

Procedure and materials. White Pekin ducklings (2-3 days of age) were divided into groups of 5 having approximately the same average weight and placed in heated cages with free access to food and water. The purified diets are fully described elsewhere(8), but those to be considered here differ only in one respect, the nature of the carbohydrate. The control group received natural foods (Purina Duck Startena and Growena). Purified diet "A" contained corn starch (Argo), diet "B" contained sucrose, and diet "C" contained commercial dextrose as the only source of carbohydrate. No striking differences in food consumption and growth rate were observed among the groups. Birds fed either the control diet or diet "A" demonstrated markedly better feathering qualities. Usually the birds were sacrificed after 30-35 days on the experimental diets. However, in one experiment it was observed that the lesion described below appeared as early as the 12th to 17th day. Sections of the pancreas were removed from 74 ducks immediately following death by decapitation and put into either Bouin's solution or a 4% solution of formaldehyde. A section of the kidney was also taken for histological study from each of 35 ducks. Paraffin sections were prepared and stained routinely with

* Supported, in part, by a grant-in-aid by The National Vitamin Foundation.

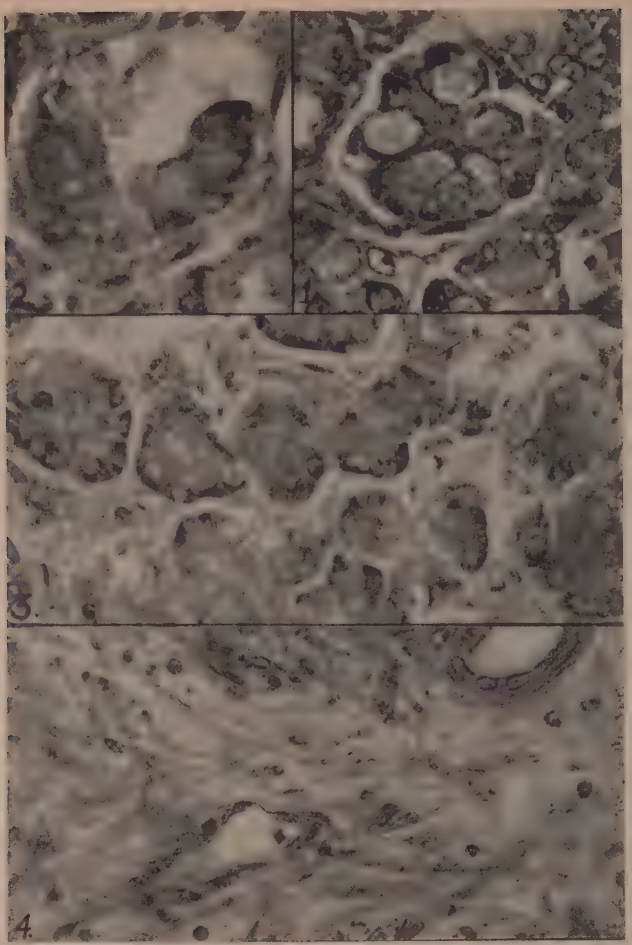

FIG. 1. Early degenerative changes in the cells of the acini, rated 1+. Hematoxylin and phloxine. × 855.
FIG. 2. More advanced changes, rated 2+. Hematoxylin and phloxine. × 855.
FIG. 3. Intralobular fibrosis, rated 3+. Hematoxylin and phloxine. × 559.
FIG. 4. Extensive pancreatic fibrosis, rated 4+. Hematoxylin and phloxine. × 559.

hematoxylin and phloxine.

Results. The pancreas from birds receiving the control diet or diet "A" appeared flesh-pink in color and was soft and pliable; it would lie over the finger like a wet string. The pancreas from birds receiving diet "B" appeared mottled-yellow to white in color; it felt hard to the touch and would hold its rigid form (as a lead pencil) when held vertically between the thumb and index finger.

The earliest microscopic changes observed in the pancreas were increased acidophilia and vacuoles within the cytoplasm of the acinar cells, pyknosis and karyorrhexis. Such lesions occurred within 12 days in ducks fed diet "B," Fig. 1. The zymogen granules coalesce to form acidophilic masses either within the degenerating cells or in the lumen of the acini, Fig. 2. These changes were usually diffuse, but occasionally focal. These degenerative changes have been rated as 1 and 2 plus in Table I.

Accompanying the degeneration of the acini there occurred a progressive increase in the

TABLE I. Relation of Diet to Incidence and Severity of Pancreatic Lesions.

Diet	Character of diet	No. of birds	Days on diet	Rating of pancreatic lesion (No. of birds)				
				Normal	1+	2+	3+	4+
Control	Natural foods	12	21	9	3			
A	Corn starch	29	30	19	8	2		
B	Sucrose	21	31	1	3	7	6	4
C	Dextrose	12	32	3	7	2		

fibrous stroma that normally separates the acini. This increase in stroma became conspicuous by the 17th day in the ducks fed diet "B." There was some variation in the quantity of stroma present in the pancreas of the different ducks, Fig. 3 and 4. The degree of fibrosis was rated 3 or 4 plus in Table I. The increased stroma apparently filled the spaces previously occupied by the acini. There was some variation in the quantity of fibrous tissue proliferation in different areas of the pancreas; a diffuse lesion was most commonly seen.

In ducks with extensive pancreatic fibrosis only a few acini could be found within the stroma, Fig. 4. The epithelial cells in these acini usually were smaller than the normal and their cytoplasm contained fewer, smaller zymogen granules. Sometimes the pancreatic capsule was markedly thickened by fibrous tissue when there was extensive fibrosis within the pancreas. It is possible that the cystic-like appearance in Fig. 4 was in reality a section through ductules or ducts. No pathologic changes were observed within the Islets of Langerhans of any duck fed the experimental rations. No significant lesions were present in the kidney of 35 ducks in which this organ was studied.

Table I is a summary of the pathologic findings in the pancreas as observed in 74 ducks. The pancreas was essentially normal in 12 ducks fed the Purina Startena and Growena control ration. Of the 29 ducks fed the corn starch diet (diet "A"), 19 had a normal pancreas; in 10 the pancreas showed early pathological changes. Of the 21 ducks fed the sucrose ration (diet "B") only one bird had a normal pancreas, while in 10 the pancreas showed extensive degeneration characterized by fibrosis. Of the 12 birds fed diet "C" 10 had essentially normal pancreases, while 2 showed a 2 plus lesion.

Discussion. The lesions in the pancreas are considered to be the result of undernutrition rather than that of any toxic effect associated with the assimilation of dietary sucrose. Some supporting evidence lies in the finding of essentially normal kidneys in all birds receiving a diet containing sucrose which demonstrates that unhydrolyzed sucrose is not entering the blood stream. This is in contrast to the observation that sucrose in the blood stream produces pathological changes in the kidney(9). Other data suggest that the severity of the pancreatic lesions was decreased when corn starch was added in increasing amounts to replace sucrose, or when certain fractions obtained during the commercial preparation of dextrose were added to diet "B." Lesions have been produced in the rat similar to those in the duck by 2 groups of workers using experimental diets(4,5) which were vastly different from each other but were similar in that the protein content of the diet was low and/or of poor quality.

It is interesting to compare pancreatic lesions observed in experimental animals to similar lesions observed in man, but one must do so with caution. It would be of considerable interest to study the nutritional history of the uremia cases reviewed by Baggenstoss (2), particularly in view of the observation that there is reasonably good correlation between vomiting and nausea, and the incidence of pancreatic degeneration which appears to accompany uremia. The pancreatic lesions described by Davies(3) and Véghelyi(4) are obviously the result of poor nutrition.

It is conceivable that the cases of pancreatic acinar tissue degeneration described by Wallace(1) and Baggenstoss(2), as well as those

described by Davies(3) and Véghelyi(4), all have a nutritional basis as a common denominator. Although it is not the purpose of this paper to discuss the similarities between this lesion and the ones found in celiac disease, fibrocystic disease of the pancreas, and sprue, it would seem worthwhile to consider a common etiology for all.

Summary. A degeneration of the acinar cells of the pancreas as the result of a nutritional deficiency has been described. The microscopic lesion was characterized by increased acidophilia and vacuoles within the cytoplasm of the acinar cells, pyknosis and karyorrhexis, which were followed by disintegration of the acinar structure and infiltration by fibroblasts. Finally, there was an increase in the perilobular and periacinar fibrous stroma and in some areas, large amounts of the parenchymal tissue were replaced by fibroblastic material. No changes were observed in the islet cells.

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Received April 8, 1952. P.S.E.B.M., 1952, v80.

Changes in Distribution of Potassium and Sodium in Rabbit Muscle Following Cold Injury.* (19558)

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Several observations may be found in the literature reporting alterations in the distribution of potassium and sodium in muscles of experimental animals following various forms of injury as prolonged ischemia, burns and trauma(1-4). Crismon and Fuhrman(5) observed, in the rabbit's muscle exposed to severe cold, a significant gain of sodium in the injured tissue ranging from 30 to 697% with an accompanying increase of extracellular water. In our experiments, the alterations in potassium and sodium distribution in relation to morphological changes in rabbit muscles subsequent to cold injury, were investigated.

Material and methods. Seven adult albino rabbits weighing 2350 to 2800 g were anesthetized with pentobarbital sodium. A standard injury was produced on one of the depilated hind legs by immersing for 4 minutes in a medium previously cooled by solid carbon dioxide to a temperature of —35°C to —37°C (6,7). The muscle biopsies for chemical analysis and cytological study were taken from anterior tibial muscle of both the exposed and unexposed leg under light ether anesthesia, using aseptic technic. The first group of animals (No. 15, 16, 17, 18) was sacrificed at 48 hours, the others (No. 1922, 2023, 2124) 24 hours after exposure to cold and portions of the same muscle were removed. The muscle tissue to be investigated was free from fat, tendons, larger blood vessels and nerves. Muscle specimens were dried to constant weight at a temperature of +105 to +110°C and digested in nitric acid. Sodium and potassium concentrations were determined by means of an internal standard flame photometer. The specimens for morphological study were preserved in Carnoy's fluid, embedded in paraffin and stained with hematoxylin and eosin and van Gieson stain.

Results in electrolyte alterations. The resulting changes are tabulated in Table I and

* This work was supported by a grant-in-aid from the Defense Research Board of Canada.

TABLE I. Redistribution of Potassium Contents in Injured and Uninjured Muscle Tissue After Exposure to −35°C to −37°C for 4 Min (mEq/100 g Dry Tissue).

	Hr after exposure								
	6 hr		24 hr		48 hr		% loss of K after		
Rabbit	Uninj.	Inj.	Uninj.	Inj.	Uninj.	Inj.	6 hr	24 hr	48 hr
15	40.30				39.52	15.92			59.8
16	43.77	31.55			47.51	11.20	27.9		76.5
17	37.87	27.77			40.48	20.5	26.9		49.3
18	39.3	20			41.57	16.99	49.1		59.2
1922	45.5	6.48	41.1	9.61			85.7	76.7	
2023	38.2	9.15	35.1	31.4			76.3	10.5	
2124	45	19.25	37.3	37.6			57.2	+.8	

TABLE II. Redistribution of Sodium Contents in Injured and Uninjured Muscle Tissue After Exposure to −35°C to −37°C for 4 Min (mEq/100 g Dry Tissue).

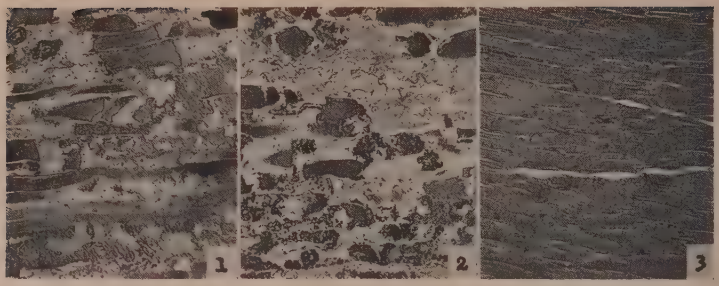
	Hr after exposure								
	6 hr		24 hr		48 hr		% gain of Na after		
Rabbit	Uninj.	Inj.	Uninj.	Inj.	Uninj.	Inj.	6 hr	24 hr	48 hr
15					6.77	55.80			724
16					10.52	103.12			879
17					11.39	54.25			377
18					8.28	72.78			779
1922	12.30	71.9	9.68	74			485	664	
2023	18.90	93	12.5	34			392	172	
2124	13.60	76.7	15.58	19.02			465	22.1	

II. All terms are expressed in mEq per 100 g dry tissue. Six hours after exposure to cold there is a significant loss of potassium from the injured tissue ranging from 26.9 to 85.7% in all experiments. On the other hand, the sodium content in the injured muscle tissue increases considerably from 392 to 485%. A similar decrease in potassium concentration can be observed in animals sacrificed 24 hours after injury, except in experiment No. 2124, in which the potassium content in the injured tissue remains unchanged as compared to the uninjured musculature. There is also a further gain of sodium in the frostbitten tissue except in the above mentioned experiment in which only a slight increase can be noted. The potassium concentration in the injured tissue decreases progressively during the 48-hour period showing a further loss up to 76.5%. At the same time, *i.e.*, at the end of the 48-hour period, the sodium content rises significantly up to values of 879%. The opposite uninjured muscle tissues in the same animal, show uniform losses in sodium concentration during the whole experimental period except in animal No. 2124, in which a slight increase can be observed. On the other hand, there is a decrease in potassium in the unexposed tissue in animals sacrificed 24 hours after cold injury. The other group of animals presents a gain in potassium in uninjured musculature 48 hours after cold injury.

Microscopic findings. The sections taken from the muscles 6 hours after exposure to cold (Fig. 1) show uniform changes consisting of primary damage to the tissue as shown by extensive degeneration of the muscle cells. It is represented by disruption, shrinkage, vacuolization and fenestration of portions or of whole cells. The cell membranes are ruptured or broken and the nuclei undergo pyknosis and karylosis. Edema fluid containing debris of sarcoplasm and nuclei distends the interstitial spaces.

More advanced degenerative changes and further disintegration of muscle cells can be seen in the sections taken 24 hours after cold injury (Fig. 2). At this time, the resorptive activity of phagocytic elements is evident and the proliferating connective tissue starts to replace necrotic muscle cells. Interstitial spaces contain less edema fluid.

Identical degenerative changes can be observed in the muscle tissue 48 hours after exposure to cold. The activity of phagocytic elements absorbing and removing the disin-



Sections taken from anterior tibial muscle at different intervals after exposure to cold. H. and E. × 72.

FIG. 1. Extensive degeneration of muscle tissues 6 hr after injury. Rabbit #2023.

FIG. 2. Further disintegration and resorption of injured cells 24 hr after injury. Rabbit #2023.

FIG. 3. Control section taken from unexposed muscle 6 hr after injury. Rabbit #17.

tegrated muscle cells predominates. Proliferating connective tissue replaces areas of the necrotic tissues. There is evidently less edema in the interstitial spaces.

In rabbit No. 2124, similar pathological alterations can be seen in the musculature. Most of the muscle fibers, however, are intact in the sections taken 24 hours after exposure to cold.

The sections taken 6, 24, and 48 hours after exposure to cold from uninjured anterior tibial muscle of the corresponding animals (Fig. 3), do not show any pathological changes in the structure.

Discussion. The exposure of living muscular tissue to cold of —35 to —37°C results in irreversible damage to the cells similar to that in trauma and burns. The injury is morphologically evidenced by breakdown of the cellular membranes and of cell protoplasm. The immediate changes appearing after injury (7-9) are most marked in sections taken from 6 to 8 hours after exposure to cold(7). At this time, the potassium concentration in the injured tissue is significantly reduced, corresponding in a degree to the microscopic findings. The loss of potassium from injured tissue increases progressively and corresponds to the concomitant disintegration of the damaged cells with the subsequent absorption of necrotic material in the course of 48 hours. A similar release of potassium from injured muscle cells has been observed in burns(1), in experimental traumatic shock(1,2), and in ischemic tissue (1,3,4), as well as in liver tissue after anoxia(10).

The distension of the interstitial spaces by edema in injured muscles indicates an accumulation of extracellular fluid in the injured region. Correspondingly, the sodium concentration of the injured tissue increases markedly. The injured tissue, however, gains more sodium than could be accounted for on the basis of increased water content of the tissue (1,4). Crismon and Fuhrman found an accumulation of more sodium in frostbitten(5) and ischemic musculature(4) than calculated on the basis of addition of extracellular fluid. This accumulation of "excess sodium"(1,4) in the injured tissue, *i.e.*, sodium not present in extracellular fluid, can be partially due to replacement of intracellular potassium by sodium(1,3,4). The combination of the loss of potassium from injured tissue and accumulation of extracellular fluid as edema in the region of injury, are partially reflected in the uninjured tissue by their increase in potassium and their decrease in sodium contents. Histologically, a decrease of edema in the injured region occurs in the course of the experiments, while a progressive rise in sodium concentration indicates a probable exchange of intracellular potassium for sodium. It seems that the redistribution of electrolytes between intra- and extracellular phase does not depend upon the amount of edema in the injured tissue. The concentration of potassium in the injured tissue depends on the number of cells involved in the process of necrosis. On the

other hand, with more extensive necrosis there is less uninjured tissue to absorb the additional potassium released from injured cells(1). More marked electrolyte changes therefore, seem to be related to the greater extent of the injury.

In rabbit No. 2124, there is an evident rise in sodium and a decrease in potassium concentration in injured tissue 6 hours after injury. After 24 hours, however, the damaged tissue shows values different from those in other experiments indicating that the analyzed tissue was limited to only a small number of injured cells, which can be confirmed in the histological sections. These experiments show the alterations in electrolyte distribution similar to the changes in electrolyte concentration in edema fluid subsequent to injury.

Finally, it is possible that the larger variations in electrolyte distribution in injured tissue might be influenced by the increased permeability of cell membranes in the adjacent, functionally damaged but morphologically intact, cells; further, varying degrees of re-establishment of circulation in injured areas and different levels of protein content in edema fluids, might accentuate or mask changes in local electrolyte concentrations.

Summary and conclusions. The redistribution of potassium and sodium in anterior tibial muscles of adult rabbits was investigated at different times after cold injury. The pathological changes were observed simultaneously by means of routine histological technics. Six hours after injury, an extensive necrosis in muscle tissue was observed with a simultaneous release of potassium from injured cells. The loss of potassium was progressive and parallel to the breakdown and subsequent disintegration of the cells during the 48-hour experiment. Conversely, the injured tissue progressively gained sodium despite the diminishing edema fluid. An increase of potassium and a decrease of sodium concentration in uninjured muscle tissues were observed. These events indicate the exchange of intracellular potassium for sodium in injured tissues. The variations in the electrolyte redistribution in injured tissues may be due to such factors as re-establishment of adequate circulation, protein content of edema fluid and ability of uninjured cells to absorb the additional potassium. The severity of the injury is apparently directly related to the electrolyte changes.

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Received March 13, 1952 P.S.E.B.M., 1952, v80.

Fluid and Electrolyte Shifts in the Hydrated Adrenalectomized Rat After Nephrectomy.* (19559)

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The normal diuresis which follows hydration is greatly impaired during adrenal insufficiency. This retention of water in the adrenalectomized rat is due in part to a decreased emptying time of the stomach and an impaired intestinal absorption of ingested water(1,2). Primarily, however, it is caused

* This investigation was supported in part by a grant from the Atomic Energy Commission.

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by an increased sensitivity of the renal tubules to an accumulating amount of an antidiuretic substance in the serum (references cited in 3). Frost and Talmage(2) have determined the sites of the retained fluid in hydrated adrenalectomized rats and correlated their observations with changes in electrolyte balance which occurred after adrenalectomy. They showed that the absorbed but unexcreted water was found to be located almost entirely in the cells. It was suggested that this tendency for water to shift to the intracellular space was caused by the loss of salt from the extracellular fluid during the previous 2-day period of adrenal insufficiency.

It was also observed that since adrenalectomized rats are unable to excrete rapidly large amounts of ingested water, more was retained for internal distribution in these animals than was retained in their controls. The present study was conducted in order to compare extrarenal fluid shifts in the hydrated control and adrenalectomized rat without the complication of renal excretion. This was accomplished by nephrectomizing the animals before hydration. An investigation of the internal partitioning of equivalent amounts of ingested water in the various experimental groups was thus made possible. An endeavor was also made to determine what extrarenal role, as suggested by other investigators(4,5), desoxycorticosterone acetate (DCA) treatment might have in altering the fluid shifts which occur in the adrenalectomized rat after hydration.

Materials and methods. Male rats weighing between 130-180 g were nephrectomized in 2 stages; the left kidney was removed 5 days before the experiment and the right one at least 2 hours before the experiment. The adrenalectomized rats also underwent a 2-stage operation; the left adrenal was removed at the same time as the left kidney, and the right adrenal was removed 2 days before the experiment. Both the control and adrenalectomized animals were starved approximately 22 hours before the experiment, and divided into non-hydrated and hydrated groups. In addition, part of the adrenalectomized hydrated rats received 2 mg of DCA subcutaneously and 1 mg intramuscularly when the last kidney was removed, and another 2 mg intramuscularly at the beginning of the experiment. Equivalent amounts of sesame oil were administered to the untreated hydrated animals. Total body water values were obtained by desiccation and the 2-hour sodium space, as measured with radiosodium^{22}, was used as an index of the extracellular fluid volume. The extra-sodium space, which afforded an index of the intracellular fluid volume, was obtained by subtraction. All fluid compartment values were made not only on the intact animal but also after the removal of the G-I tract in order to determine the location of the ingested water which had been absorbed. Serum sodium and potassium values were determined by means of a Beckman Flame Spectrophotometer according to the method described by Mosher *et al.*(6). Total sodium in the 2-hour sodium space was obtained by multiplying the serum sodium concentration by the sodium space. Details of the methods have been previously described(2).

Results. The fluid and electrolyte shifts, which occurred in the nephrectomized non-hydrated adrenalectomized rats, followed both quantitatively and qualitatively those changes which have been reported previously for non-nephrectomized animals(2). As is indicated in Tables I and II, the total body water of the non-hydrated animals did not change after adrenalectomy. The sodium space decreased and a significant shift of fluid to the cells was indicated by an increase in the extra-sodium space. Electrolyte changes in the non-hydrated animals are shown in Table III. The expected rise in serum potassium and decrease in serum sodium after adrenalectomy were observed. There was also a 10% loss of sodium from the 2-hour sodium space. This loss of extracellular sodium apparently caused the observed shift of fluid to the cells. Changes in the gutless non-hydrated rats paralleled those of the intact animals.

As is shown in Table IV the hydrated adrenalectomized animals withheld over 3 times as much of the ingested water in their G-I tracts as did their controls. This difference was due to both a decreased emptying time of the stomach and to an impaired intestinal absorption. Although it was not significantly established, DCA treatment tended to correct

TABLE I. Distribution of Water in Hydrated, Control and Adrenalectomized Rats after Nephrectomy.

		No.	Total body water, cc/100 g ± S.E.	Expected total body water, cc/100 g	Sodium space, cc/100 g ± S.E.	Water retained in sodium space, cc/100 g	Extra-sodium space, cc/100 g ± S.E.	Water retained in extra-sodium space, cc/100 g
Control rats	No water	11	70.9 ± .4	—	33.3 ± .7	—	37.6 ± .5	—
	Water	13	79.9 ± .4	80.3	37.8 ± .4	4.5	42.1 ± .4	4.5
Adrenalecto-mized rats	No water	11	70.8 ± .4	—	30.9 ± .4	—	39.9 ± .3	—
	Water	12	79.8 ± .2	80.1	35.5 ± .6	4.6	44.3 ± .6	4.4
	Water & DCA	12	80 ± .4	80.1	35.9 ± .6	5	44.1 ± .5	4.2

TABLE II. Distribution of Absorbed Water as Determined in Gutless Nephrectomized Rats.

		No.	Total body water, cc/100 g ± S.E.	Water absorbed from the G-I tract, cc/100 g	Sodium space, cc/100 g ± S.E.	Water retained in sodium space, cc/100 g	Extra-sodium space, cc/100 g ± S.E.	Water retained in extra-sodium space, cc/100 g
Control rats	No water	11	70.2 ± .4	—	32.6 ± .7	—	37.6 ± .5	—
	Water	13	78.4 ± .5	8.2	36 ± .5	3.4	42.4 ± .5	4.8
Adrenalecto-mized rats	No water	11	69.7 ± .5	—	29.6 ± .5	—	40.1 ± .6	—
	Water	12	74.5 ± .4	4.8	30.7 ± .6	1.1	43.8 ± .7	3.7
	Water & DCA	12	75.3 ± .7	5.6	32.1 ± .8	2.5	43.2 ± .4	3.1

TABLE III. Electrolyte Determinations in Control and Adrenalectomized Nephrectomized Rats.

		Serum K,		Serum Na,		Total sodium in 2-hr sodium space, in mEq/100 g ± S.E.	
		No.	mEq/l ± S.E.	No.	mEq/l ± S.E.	No.	Intact	Gutless
Control rats	No water	11	3.95 ± .11	11	139 ± .7	11	4.63 ± .07	4.53 ± .10
	Water	13	4.72 ± .19	13	126 ± 1.3	13	4.77 ± .06	4.53 ± .07
Adrenalecto-mized rats	No water	11	6.53 ± .21	11	134.7 ± 1.1	11	4.15 ± .05	3.99 ± .06
	Water	9	6.83 ± .39	12	122.7 ± 1.1	12	4.36 ± .07	3.78 ± .06
	Water & DCA	9	5.69 ± .24	12	122.5 ± .7	12	4.39 ± .10	3.93 ± .10

this delayed intestinal absorption.

Distribution of the ingested water in animals with intact G-I tracts is indicated in Table I. The total body water of both the hydrated, control and adrenalectomized rats was very close to the expected value calculated on the basis of retained water. Approximately one-half of the ingested water was contained in the extra-sodium space of all the animals regardless of experimental treatment. Water which is retained in the lumen of the G-I tract is still part of the sodium space. Since almost twice as much water was absorbed from the intestines of the controls as compared with the adrenalectomized animals, it would appear that the sodium space of the control group should have been proportionally smaller. The reason it was not smaller was because percentage-wise less of the absorbed water actually shifted to the extra-sodium space in the controls as compared with the adrenalectomized group. Therefore the lack of difference between the controls and adrenalectomized groups concerning the internal partitioning of ingested water was more apparent than real.

To determine the sites of absorbed water, it was therefore necessary to measure the fluid compartment values of the nephrectomized rats after the G-I tract had been removed (Table II). In the hydrated gutless

TABLE IV. Retention of Water in Hydrated, Control and Adrenalectomized Rats after Nephrectomy.

		No.	Total water retained in the animal, cc /100 g ± S.E.	Total water retained in the G-I tract, cc/ 100 g ± S.E.	Total water absorbed from the G-I tract, cc/ 100 g ± S.E.
Control rats	Water	13	9.4 ± .1	1.5 ± .2	7.9 ± .3
Adrenalectomized rats	Water	12	9.3 ± .1	4.9 ± .2	4.4 ± .2
	Water & DCA	12	9.3 ± .1	4.3 ± .5	5 ± .5

animals, the difference between the non-hydrated and hydrated total body water values afforded another index of the amount of water absorbed from the G-I tract. Within the limits of experimental error, the results are in good agreement with the values for total water absorbed which are given in Table I. It was also noticed by this method of determination that DCA again appeared to enhance the absorption of water from the G-I tracts of the adrenalectomized animals.

In all the experimental groups more of the absorbed water was found to be located in the extra-sodium space than in the sodium space. This was particularly true for the adrenalectomized rats in which over three-fourths of the absorbed water shifted to the cells. Less than 60% of the absorbed water shifted to the extra-sodium space of the controls. DCA treatment appeared to prevent this increased shift of fluid to the cells in the hydrated adrenalectomized animals, for the percentage distribution of absorbed water between the sodium and extra-sodium space was returned essentially to normal. Additional experiments, however, would be necessary to confirm this hormonal action.

The changes in electrolyte balance during the 2-day period of adrenal insufficiency suggest the reason for the fluid shifts observed in the hydrated adrenalectomized rats (Table III). As would be expected, hydration after nephrectomy did not significantly alter the amount of total sodium in the 2-hour sodium space of either the control or experimental animals. However, the low serum sodium values of the adrenalectomized rats were reduced still further by dilution after hydration. More of the absorbed water shifted to the cells of these animals than it did in the controls probably because the loss of salt from the extracellular fluid during the 2-day period of adrenal insufficiency resulted in a higher osmotic gradient.

Thus it is apparent that the fluid and electrolyte shifts which occurred in both the control and adrenalectomized hydrated nephrectomized rats are in agreement with the results which were reported earlier for non-nephrectomized animals(2).

Although changes in serum potassium were followed in these investigations merely as an index of adrenal insufficiency, several unexpected observations were made during the course of the experiments. Serum potassium unlike sodium was not diluted upon hydration and in fact, under some experimental conditions it actually increased. Also, there was some indication that adrenal insufficiency tended to exaggerate this phenomenon.

Possibly hydration may act in the same way as other stressing agents by inducing a general systemic release of intracellular potassium. If, as has been suggested(7), the release of potassium during the shock phase of the alarm reaction is conditioned in part by a state of relative cortical insufficiency, one would expect that these changes in serum potassium would be exaggerated after adrenalectomy.

Summary. Normal and 2-day adrenalectomized rats were nephrectomized and subsequently hydrated, thus making it possible to study the internal partitioning of relatively large amounts of ingested water. There was a delayed intestinal absorption of water in the adrenalectomized animals. A larger percentage of absorbed water shifted to the cells of these animals than was observed in the controls apparently as a result of salt depletion during the previous 2-day period of adrenal insufficiency. Although there was a tendency for desoxycorticosterone acetate therapy to enhance intestinal absorption and also to pre-

vent the excessive intracellular hydration of the adrenalectomized animals, these extrarenal hormonal actions were not significantly established. Alterations in potassium metabolism were also briefly discussed.

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Received May 6, 1952. P.S.E.B.M., 1952, v80.

Cortisone and Action of Exogenous Thyrotrophin in Hypophysectomized Rats.* (19560)

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Several workers have reported a depression of thyroid function by cortisone in man and in the rat(1-8). A block of the thyrotrophic function of the pituitary was suggested as a possible explanation of this phenomenon (5,6). Perry(4), however, presented data which are not in agreement with this concept and we made observations(9) which appeared to indicate that pituitary thyrotrophin (TSH) output is enhanced rather than depressed by cortisone. Woodbury *et al.*(10), on the other hand, described a striking modification of the action of exogenous TSH in hypophysectomized rats by cortisone. They found that the TSH induced increase in the thyroid I^{131} uptake of their animals was partly prevented by simultaneously administered cortisone. Since these findings do not necessarily indicate an interference of cortisone with TSH action *per se,* the present study was undertaken to assess the influence of the steroid on the effectiveness of exogenous TSH in hypophysectomized rats, using the change in thyroid cell height as the index of thyrotrophic stimulation. This technic has been used previously by Griesbach and Purves(11) for assay of TSH and recent observations by Ghosh *et al.*(12) have also shown a roughly rectilinear relationship between thyroid cell height increment and log dose of TSH administered to hypophysectomized rats.

Method. Hypophysectomized male rats weighing between 130-145 g were purchased from the Hormone Assay Laboratories, Chicago. Twelve days after the operation they were divided into 4 groups. Group I (14 rats) received no treatment, group II (15 rats) was injected with 5 mg of cortisone† daily for 10 days, group III (14 rats) received daily injections of .3 mg TSH‡ for 10 days, while group IV (16 rats) was treated for the same length of time with both cortisone and TSH in the aforementioned dosages. The rats were fed Purina Laboratory Chow supplemented with horse meat and fresh oranges. All treated animals, as well as the controls, were sacrificed 24 hours after the last injection. The thyroids were weighed on a torsion balance, fixed in SUSA, imbedded in paraffin and cut at 5 μ. Sections running through the center of the gland were stained with Gomori's trichrome method. The slides were projected and the height of one cell per follicle was measured in 50 follicles picked at random from each gland.

* Supported by a gift from the American Cancer Society and a grant from the Central Scientific Fund, State University of Iowa College of Medicine.

† Cortisone (Cortone acetate Merck) was generously supplied by Dr. Elmer Alpert of Merck and Co., Rahway, N. J.

‡ TSH (Parke Davis #50P4) was made available through the courtesy of Dr. D. A. McGinty and Mr. W. Donaldson of Parke, Davis & Co., Detroit, Mich.

TABLE I. Thyroid Weights and Average Follicular Cell Heights in Hypophysectomized Rats Given Cortisone, TSH or Cortisone + TSH for 10 Days.

	Thyroid wt, mg	Avg cell height ± stand. error, μ
Controls	6.9	4.1 ± .18
5 mg cortisone daily	7.7	4.3 ± .10
.3 mg TSH daily	8.8	8 ± .15
5 mg cortisone + .3 mg TSH daily	8.4	9.1 ± .22

Results. The thyroid weight response to TSH (Table I) was not marked and its statistical analysis was considered meaningless since a painstaking dissection of the glands was not undertaken in order to avoid histological artefacts due to prolonged handling. The average thyroid cell height (Table I) was almost doubled by TSH. Cortisone in itself had no morphological effects on the cells of the thyroid follicles. The effectiveness of TSH on thyroid cell height, far from being inhibited, actually appeared to be slightly but significantly ($P < .01$) enhanced by simultaneously given cortisone.

Discussion. The findings reported in this paper support the conclusion of Perry(4) that cortisone does not act as a thyroid depressant by inhibiting the action of TSH. They also suggest that the data of Woodbury *et al.*(10) regarding the interference of cortisone with the increase in thyroid I^{131} uptake, which exogenous TSH brings about in hypophysectomized rats, should not be interpreted as a blocking effect of the steroid on TSH action itself. Our studies on cortisone treated intact rats(9) pointed to an increased release of TSH consequent to cortisone administration and the possibility that cortisone might act as a mild goitrogen was considered. The observation that cortisone apparently may increase the morphological response of the thyroid cells of hypophysectomized rats to TSH is compatible with the idea of an antithyroid action of cortisone, since a pronounced potentiation of TSH action by thiouracil has been encountered previously in a histological study on hypophysectomized rats(13).

Summary. 1. Groups of hypophysectomized rats were given 5 mg of cortisone daily, .3 mg of TSH daily, and 5 mg of cortisone + .3 mg TSH daily for 10 days. Measurements of thyroid cell height were made. 2. The average thyroid cell height was 4.1 μ in the hypophysectomized controls, 4.3 μ in the cortisone treated rats, 8.0 μ in the rats treated with TSH, and 9.1 μ in the animals which received both TSH and cortisone. 3. It is concluded that cortisone, under the conditions of the experiment, did not block and probably even slightly enhanced the effect of TSH on the thyroid.

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Received May 1, 1952. P.S.E.B.M., 1952, v80.

Occurrence of Jaundice in Inoculated Suckling Mice.* (19561)

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Laboratories engaged in studying the Coxsackie viruses commonly use many litters of suckling mice. This preliminary account notes the previously unreported appearance of jaundice in such mice following inoculation of several bacteria-free filtrates of suckling mouse carcasses. The phenomenon is apparently not due to a transmissible agent and its mechanism remains to be elucidated.

Ten percent extracts of human feces were centrifuged for 10 minutes at 2000 r.p.m. and 1000 units of penicillin G and 500 units of streptomycin per ml added. Newborn mice (Harlan albino stock) were inoculated subcutaneously with 0.03 ml each of fecal extracts. From 12 apparently negative fecal specimens successive serial "blind-passages" were employed. Three separate lines of such "blind-passages" have yielded pools of infant mouse carcasses capable of producing jaundice upon subcutaneous inoculation. These 3 separate pools of bacteria-free filtrates (Corning sintered glass UF filters used) have been studied in mouse litters only. Following the subcutaneous inoculation of 0.03 ml of icterogenic filtrate the recipients appear normal until 36 to 48 hours has elapsed. Jaundice of varying depth then appears; from the plasma of deeply jaundiced baby mice a total bilirubin of 7.4 mg % has been found. The majority of deeply jaundiced mice die within 12 to 24 hours.

Ability to produce this jaundice falls off rapidly with advancing days of life, and is most easily induced if inoculation is carried out on the day of birth (Table I).

TABLE I. Influence of Age on Development of Jaundice.

	Age at inoc.				
	Newborn	1 day	2 days	3 days	4-7 days
No. inoc.	275	46	26	47	34
No. jaundice	182	23	10	17	0
%	66	50	38	36	0

Bilirubin is present in both urine and feces during the jaundice. The peripheral blood shows evidence of a hemolytic process when compared with stain smears of control mice injected with nonicterogenic mouse carcass suspensions. Thus polychromatophilia, reticulocytosis and the presence of target cells is marked in degree. Hepatic sections have failed to reveal changes other than the extramedullary hematopoiesis normally found in infancy.

It has not been possible to transmit jaundice serially to other litters by the inoculation of liver, brain or whole carcass suspensions of jaundiced mice. Suckling animals other than mice have not been studied.

The rather striking influence of age of the recipient mice suggests that immaturity of the liver is an important factor in this apparently non-infectious form of jaundice.

Summary. Jaundice has been observed in baby mice inoculated with bacteria-free filtrates of whole carcass suspensions of suckling mice. An hemolytic anemia is associated with the jaundice; mice more than 4 days of age at time of inoculation appear resistant. There is no evidence that the phenomenon is caused by a primary infectious agent.

* Aided by a Grant from The National Foundation for Infantile Paralysis.

Received April 14, 1952. P.S.E.B.M., 1952, v80.

Isolation of a New Infectious Agent from the Vesicles of Chicken Pox. (19562)

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During the course of an effort at isolation of an etiological agent from chicken pox, a new organism was recovered on 4 occasions and while the relationship of this agent to human disease is not clear, its pathogenic qualities in guinea pigs and embryonated eggs were thought to be of sufficient interest to justify a brief description of the findings.

The starting material for all 4 isolations was vesicular fluid from early typical cases of chicken pox (1949-50). Usually the fluid from several patients was pooled and stored on dry ice before use. Two distinct methods of isolation were successful and yielded 2 strains each. For the first method, about 0.05 ml of vesicular fluid was added to a small piece of infant foreskin and the latter was minced with scissors and suspended in Tyrode's solution containing 10% bovine serum ultrafiltrate. After 4 days, the supernatant fluids were removed and passed at 3- to 4-day intervals on the dropped chorioallantoic membrane (CAM) of 11-day-old chick embryos. A series of 4 blind passages were negative. To the fourth passage CAM suspensions were added the original tissue culture materials which had been ground in their suspending fluids and further blind passages on the CAM were continued. In one case after a total of 6 and in the other of 9 embryo passages, definite pock formation was noted. The lesions on the CAM were small, opaque and discrete elevations, often extending beyond the inoculated portion of the membrane, a typical example of which is shown in Fig. 1. After their initial appearance, pocks developed regularly in serial passage and could be counted as a rough means of measuring the infective titer of a preparation.

The second pair of strains was isolated by inoculating vesicular fluid intraperitoneally into 250 g guinea pigs and passing a suspension of stripped peritoneum at 3- to 4-day intervals by the same route. Each peritoneal suspension was tested for pock formation by

FIG. 1. CAM of 12-day-old egg inoculated with VF strain at 9 days.

a single passage on the CAM. In one of these series, no animal had any symptoms and there was no evidence of the presence of a pock forming organism through 6 passages. The 7th passage animal had a temperature of 104°F, but the peritoneum was negative on the CAM. Two animals in the 8th passage received this material. One of these had no symptoms or fever and although its peritoneum contained a low titer of pock forming organisms, attempts to propagate this agent in series failed. The second animal in the 8th passage was very sick, had fever and a pock titer on the CAM of $3X10^6$. The agent from this animal was regularly propagated in further animals with the production of fever and peritonitis. In the second guinea pig series, symptoms and fever appeared on the

4th and 5th passages, but the organism was first recovered from only one of 4 animals in the 6th passage, and was propagated regularly in further series. These strains were similar to the VF tissue culture strain in every way tested. It is obvious from an examination of the 2 guinea pig series that the ability of these strains in unadapted form to multiply was quite variable in different animals and uniformly successful isolations by this method could not be expected.

Over a dozen other chicken pox fluids, as well as material from other human skin lesions, were tested by the foregoing methods without obtaining any detectable infectious agent. The reasons for this failure are not clear.

One of the 4 strains (VF) isolated was studied intensively. The organism involved had a highly pleomorphic cocco-bacillary form and stained purple with Giemsa and red with Machiavello's stain. It ranged up to about 1.5 μ in length and filtration and centrifugation studies were consistent with this size. The organisms were best seen in tissue sections where they appeared in large densely packed colonies within the cytoplasm of cells. On storage at either 4°C or on dry ice, the viability of preparations dropped rapidly unless 20% glycerine was added. Extensive attempts to cultivate the organism in artificial media, including those which are optimal for the pleuropneumonia and hemobartonella groups, were without success.

Host range. The VF strain, once isolated on the CAM, was virulent in several hosts without any further adaptation. These hosts were as follows: 1) *The chorioallantoic membrane.* The chorionic side of the CAM provided excellent conditions for growth and the organism reached maximum titer in 3 days. Suspensions of heavily infected membranes yielded pock counts up to 1×10^6 per g of tissue. Embryos ordinarily survived infection of the dropped membrane and the organism did not spread to other organs. 2) *Chick embryo yolk sac.* Multiplication was rapid after yolk sac inoculation and the embryos died 4 to 9 days after infection, depending on the size of the inoculum. Some yolk sacs produced 5×10^7 pocks per g. Tissue sections showed the organisms to be almost entirely within the cytoplasm of yolk sac cells. 3) *Guinea pig peritoneum.* The inoculation of as little as 0.5 ml of a 10^{-6} dilution of infected yolk sac into the guinea pig peritoneum produced a febrile response after an incubation period of 6 to 7 days. The injection of larger amounts produced a more immediate response with temperatures of 105°F and over, and lasting 5 to 6 days. With 1 to 10% yolk sac, the animals often died after 3 to 4 days with quite variable pathologic findings. In some the peritoneum appeared normal while in others there was a diffuse fibrinopurulent but bacterially sterile exudate. The spleen and scrotum were not involved except in overwhelming infections. The peritoneum contained the highest titer of organisms, but this was usually less than that of the CAM. 4) *Guinea pig lung.* The organism when given intranasally produced pulmonary consolidation, fever and, with large inocula, death. The yield of infective agent from such lungs was far better than from any other organ of the guinea pig and lung could be used as a source of complement fixing antigen. Lung involvement was common following inoculation by other routes as well. 5) *Guinea pig brain.* A lethal effect could be obtained by the intracerebral inoculation of a large number of organisms. The cellular reaction was largely in the meninges and the organism did not multiply to high titer. There was no paralysis. 6) *Rabbit eye.* Organisms were demonstrated morphologically and by pock count in the cornea and nictitating membrane of rabbits after inoculation of the scarified surface with infective material. There was also clouding and focal opacity of the cornea. No evidence of multiplication was obtained after the inoculation of the VF strain into the amniotic and allantoic sacs of the chick embryo. Repeated attempts at passage in mice by several routes were all negative. Voles and hamsters also gave negative results.

Immunological behavior. Active immunity to infection in the guinea pig was not striking in that animals that had recovered from an initial severe infection, often had a febrile response, equivalent to that of controls on reinoculation. However, active immunity could readily be demonstrated by the manner in which recovered animals handled a second

TABLE I. Cross Complement Fixation Test Between Various Rickettsiae and the VF Strain.

Immune serum	Dilution of VF antigen 1/1	1/2	1/4	1/8	1/16
VF	4+	4+	4+	4+	—
Murine typhus	—	—	—	—	—
Q-9 mile	—	—	—	—	—
Rocky Mt. spotted fever	—	—	—	—	—
Rickettsial pox	—	—	—	—	—
Boutenneuse fever	—	—	—	—	—
S. African tick fever	—	—	—	—	—
C. Maculatum	—	—	—	—	—

This test was performed with 8 units of the various immune sera (guinea pig) and two-fold dilutions of the VF antigen. This test was done by Dr. Sam C. Wong of Lederle Laboratories, Pearl River, N. Y.

inoculum by the intranasal route. Guinea pigs that had recovered from an intraperitoneal infection were challenged with 10% CAM intranasally. These animals developed fever and signs in the same proportion as the controls, but after 3 days it was very difficult to find organisms on direct smear and only a few lungs yielded pocks in low titer on the CAM. Large numbers of organisms were seen in control smears and pock counts were one to 5 million per gram. Neutralization tests were performed in a number of ways but in no instance did serum from hyperimmune guinea pigs give any trace of a neutralizing effect, either in reducing the pock count on the egg membrane, in prolonging survival after yolk sack inoculation, or in alleviating any of the pathological effects in guinea pigs. An excellent complement fixing antigen was prepared from yolk sac by the method of Topping(1) in which the antigen sedimentable at 5000 rpm is rendered soluble with ether extraction. Immune guinea pig sera reacted with this antigen when diluted as much as 1:512. The titer of guinea pig sera after a single infection was low, often below the limit of detectability but this usually rose to very high levels within a week after re-infection. CF tests were performed with the VF antigen and sera containing antibodies against various Rickettsiae (Table I). No cross relationships were found. CF tests were also done with chicken pox vesicular fluid and the VF strains as antigens against acute and convalescent chicken pox sera and immune guinea pig sera (VF) with the results shown in Table II. There was no demonstrable antigenic relationship of strain VF to chicken pox. Convalescent sera were obtained from several hundred individuals who had fevers either of obscure origin or were suspected of having a virus or Rickettsial infection. They were tested for complement fixation with the VF antigen with negative results. The possible significance of this is somewhat obscured by the poor CF titers found in guinea pigs after a single infection.

Effect of antibiotics on infection. The effect of antibiotics on VF infection was determined by comparing the pock count in embryos pre-treated with the drug with the count in untreated eggs. Moderate but significant reductions in pock count were obtained with aureomycin and chloromycetin at a level of 500 μg per egg. A similar reduction was obtained with terramycin at 5 μg per egg. Streptomycin at 5 μg per egg and sulfadiazine at 5 mg per egg consistently reduced the pock count over 90%. In yolk sac infections, PABA had a slight effect at 1 mg per egg but at 5 mg the mortality was reduced to zero. Continued treatment over a period of one week with streptomycin and sulfadiazine significantly reduced the mortality in guinea pigs given massive intraperitoneal infections.

Discussion. Inasmuch as no satisfactory method of isolating organisms like the VF strain has been worked out, it is at present impossible to say with assurance where such strains occur in nature. If these strains were isolated as a result of activation of a latent agent, then human skin, the guinea pig and chick embryos would be the most obvious sources. However, the only common factor in the two types of isolation was the chick embryo and it is thought that this is a highly unlikely source, since in the guinea pig series the transfer of peritoneum to the CAM at the critical stage resulted in overwhelming pock formation on initial CAM passage. Barring some obscure form of accidental contamination of each isolation series, the vesicular fluid remains as the most likely source of the agent. Since it plays no obvious direct role in chicken pox, perhaps the organism is latent in the human skin.

A final point of interest concerns the classi-

TABLE II. Cross Complement Fixation Test Between Chicken Pox and VF Antigens.

		Serum dilution						
		8	16	32	64	128	256	512
Chicken pox, acute phase	Vesicular fluid 1/20	—						
Chicken pox, convalescent	"	4+	4+	4+	4+	2+	±	
Guinea pig, normal	"	—	—	—	—	—	—	
Guinea pig, immune VF strain	"	—	—	—	—	—	—	
Chicken pox, acute phase	VF-Guinea pig lung	—	—	—	—	—	—	
Chicken pox, convalescent	"	—	—	—	—	—	—	
Guinea pig, normal	"	—	—	—	—	—	—	
Guinea pig, immune VF strain	"	4+	4+	4+	4+	4+	4+	±

The human sera were taken during the acute and convalescent stage of a typical case of chicken pox and the immune guinea pig serum was a pool from animals that had received several inoculations of egg membrane material. The vesicular fluid antigen was fresh fluid from vesicles of an acute case of chicken pox diluted 1:20, and the VF antigen was prepared from the lungs of intranasally infected guinea pigs used in the test at a concentration of 5%.

fication of this new organism. It resembles the Rickettsiae much more closely than any other group of microorganisms. The more obvious differences from this group lie in that area where present knowledge of the new strain is incomplete, such as information about possible insect vectors and natural hosts. In addition, it shows no serological cross relationships with a number of the known pathogenic Rickettsiae. On the other hand, the organism is strikingly similar to this group in a number of other respects, such as size, morphology, staining reactions, failure to grow in cell-free media, host range, insusceptibility to neutralization, and in having an intracellular site of multiplication. Its antibiotic spectrum is not too dissimilar from other Rickettsiae and in forming pocks on the chorioallantoic membrane it is quite similar to *Rickettsia burneti* (2). The behavior of the VF strain was quite unlike the organism obtained by Tatlock (3) from guinea pigs. We were unable to obtain this strain for direct comparison.

Summary. Four strains of a Rickettsia-like agent were isolated by two methods from chicken pox vesicular fluid. These agents produced pocks on the chorioallantoic membrane, were pathogenic for guinea pigs by several routes and appeared to grow only within the cytoplasm of cells. Their origin and relationship to human or other naturally occurring disease is obscure.

The authors take pleasure in acknowledging their indebtedness to the staffs of Willard Parker Hospital, New York Skin and Cancer Hospital and the nursery staff of Presbyterian Hospital for their assistance in collecting material. We are particularly indebted to Miss Olga Simonovic of the Bureau of Laboratories, New York City, and to Dr. Sam C. Wong of Lederle Laboratories for performing complement fixation tests and checking many of our results.

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Received April 28, 1952. P.S.E.B.M., 1952, v80.

Free Estrone in Urine (False Positive Richardson Pregnancy Test) In Association with Ovarian Tumors. (19563)

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(Introduced by A. J. Goldforb.)

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Richardson(1) has recently described a chemical test for pregnancy, based upon the identification of free estrone in the urine by a color reaction with 2,4-dinitrophenylhydrazine. A high degree of accuracy has been claimed for this test(1,2). The test, as described, is simple, rapid, and economical. If it were as accurate as claimed, it would have many advantages over the usual biological test methods.

It was, therefore, decided to run the chemical method in comparison with the 2-hour rat test(3-6), based on rat ovary hyperemia reaction to chorionic gonadotropin, augmented by a pituitary synergist.

Methods and results. The following report is based on 100 consecutive cases, examined by the two methods. All urines used were first morning specimens. In the 100 cases studied, 15 were negative by both tests; 66 were positive by both; 14 were positive by the chemical method, negative by the biological; 5 were negative by the chemical, positive by biological test. This gives an over-all accuracy of 81%, with 17.5% false positives.

The disagreements are listed in Table I. In 10 of the 14 cases, in which positive chemical tests and negative biological tests were obtained, ovarian masses were clinically suspected. The pregnancy tests had been ordered originally to rule out the possibility of pregnancy before abdominal exploration. In 8 of these 10 cases, ovarian tumors were found at operation. In 2 others, ovarian tumor is still

TABLE I. Discrepancies Between Chemical and Biological Tests.

Patient	Age	Pregnancy test Chem.	Pregnancy test Rat	Clinical symptoms and/or diagnosis	Pathological findings	Confirmation of pregnancy
S.	35	+	—	Amenorrhea 1 mo. Pain in abdomen	Pseudomucinous cystoma	Hysterectomy; not pregnant
U.	24	+	—	Backache, nausea, vomiting – few days	Dermoid cyst	At operation: small, non-pregnant uterus
L.	44	+	—	? Myoma uteri or ovarian mass	Bilateral papillary serous cystadenocarcinoma of ovaries, with metastases	Clinically not pregnant
B.	33	+	—	Ovarian cyst	Bilateral dermoid cyst	Clinically not pregnant
D.	27	+	—	Palpable "	Parovarian cyst	Curettage showed proliferative endometrium. Not pregnant
J.S.	31	+	—	Pain in L.L.Q. Palpable ovarian mass	Ovarian "	Previous bilateral tubal ligation. Clinically not pregnant
C.	21	+	—	Abdominal mass, 3 mo	Serous papillary cystoma	Clinically not pregnant. Bilateral chronic salpingitis
G.	27	+	—	Palpable cyst on routine examination	Dermoid cyst	Clinically not pregnant
Z.	26	+	—	Amenorrhea since last pregnancy 7 months before. Enlarged ovary by palpation	Not operated	Clinically not pregnant

Patient	Age	Pregnancy test Chem.	Pregnancy test Rat	Clinical symptoms and/or diagnosis	Pathological findings	Confirmation of pregnancy
R.	24	+	—	Questionably palpable enlarged ovary	" "	Previous bilateral tubal ligation. Clinically not pregnant
D.L.	28	+	—	3 mo irregular uterine bleeding; wt loss; hyperthyroidism	Thyroidectomy: hyperplasia	Clinically not pregnant
M.G.	22	+	—	Nausea and vomiting L.M.P. 3 wk before. Acute gastritis		
O.	42	+	—	Amenorrhea		
C.S.		+	—	Uterine bleeding. Received diethylstilbestrol and ant. pituitrin within 24 hr of urine collection		
L.B.		—	+		Curettage 11 days before: viable placental tissue	
M.Z.		—	+	L.M.P.—6 wk before		
M.C.	43	—	+	L.M.P.—5 " "		Clinically pregnant
A.		—	+	L.M.P.—6 " "		
L		—	+	L.M.P.–12 " "		

clinically suspect, but as yet unproven.

The finding of false positive chemical tests for pregnancy in patients having ovarian tumors is both unexpected and of interest. Among the false positive cases reported by Merkel(2) was one with ovarian carcinoma. None of the tumors examined was of the endocrine-functioning types. The tumors represent a considerable variety, but share in common only the replacement of ovarian tissue. It may be that there are associated disturbances of pituitary gonadotropin production with high levels of circulating estrogen, which appears in the urine as free estrone, thus giving a positive chemical test for pregnancy. Possible explanations are under investigation. The reversal to negative following oophorectomy has been studied so far in only one case. A weakly positive chemical test was obtained 7 days after operation; 4 days later it was negative.

Summary and conclusions. 1. The chemical pregnancy test of Richardson and the rat ovary hyperemia test were run simultaneously on 100 consecutively submitted first morning urine specimens. 2. 81% concordance was found. There were 17.5% false positive chemical tests, 25% false negative. 3. Of the 14 false positive cases, 10 gave clinical evidence of ovarian tumor; 8 of these were confirmed by surgical excision and pathological examination; the other 2 have not been explored. 4. The chemical pregnancy test of Richardson is not sufficiently specific to permit of its general use in the diagnosis of early pregnancy.

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Received May 5, 1952. P.S.E.B.M., 1952, v80.

ERRATUM

Article 19463, Growth-Promoting Activity of L-lyxoflavin. Line 19 should read "activity of riboflavin for *Lactobacillus casei.*"